IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Rajiv SHAH, et al.
Title:	METHOD FOR FORMULATING A GLUCOSE OXIDASE ENZYME WITH A DESIRED PROPERTY OR PROPERTIES AND A GLUCOSE OXIDASE ENZYME WITH THE DESIRED PROPERTY
Appl. No.:	10/715,143
Filing Date:	11/17/2003
Examiner:	Yong D. Pak
Art Unit:	1652
Confirmation Number:	1899

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This communication is an Appeal Brief, responsive to the Final Office Action dated August 7, 2008, concerning the above-referenced patent application.

Under the provisions of 37 C.F.R. § 41.37, this Appeal Brief is being filed with the appropriate appeal fee under 37 C.F.R. 41.20(b)(2). An appeal fee of $500.00 was submitted with Applicant's Appeal Brief dated August 15, 2007. Instead of filing an Answer in response to that Appeal Brief, the Examiner re-opened prosecution by issuing the Office Action dated November 14, 2007. In that Office Action, the Examiner stated that the previously paid notice of

appeal fee and appeal brief can be applied to a new appeal (Office Action dated November 14, 2007, pg. 2 ll. 7-9.)

Accordingly, Applicant requests that the previously paid fees for filing the Appeal Brief dated August 15, 2007, be applied to the present Appeal Brief. If that fee is deemed to be insufficient, authorization is hereby given to charge any deficiency (or credit any balance) to the undersigned deposit account 19-0741.

I. REAL PARTY IN INTEREST

The real party in interest for the above referenced patent application and the present Appeal is the assignee of record for the above referenced patent application, Medtronic Minimed, Inc., as recorded at Reel 012818, Frame 0025.

II. RELATED APPEALS AND INTERFERENCES

Except as discussed in this section, Applicant is not aware of any interferences or legal proceedings that would have a bearing on the Board's decision in the present Appeal.

The present application is a Divisional application of U.S. Application No. 10/035,918. An appeal brief has been filed for U.S. Application No. 10/035,918 dated September 23, 2008, no decision has been rendered in that Appeal.

The present application also claims the priority filing date of U.S. Provisional Application No. 60/335,585 (now expired), for which no substantive examination on the merits was conducted by the U.S. Patent and Trademark Office.

III. STATUS OF CLAIMS

Claims 1-19 are pending in the application. However, claim 18 has been withdrawn from consideration by the Examiner. Accordingly, claims 1-17 and 19 are pending and under consideration in the present application. Each of those claims is included in at least one of the rejections under general grounds identified in the Final Office Action and discussed in Sections

VI. and VII., below. The present appeal relates to each of the rejections and, thus all of the rejected claims (i.e., claims 1-17 and 19).

IV. STATUS OF AMENDMENTS

No amendments have been filed, subsequent to the Final Office Action of August 7, 2008.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Embodiments of the present invention relate, generally, to a method employing directed evolution techniques for formulating a glucose oxidase enzyme having peroxide-resistant characteristics for use, by way of example, in a sensing device.

An example implantable sensing system contains a sensing device that is inserted into a vein, an artery, or any other part of a human body where it could sense a desired parameter of the implant environment. An enzyme may be placed inside of the sensing device and employed for sensing. If the device is a glucose-sensing device, then a combination of glucose oxidase (GOx) and human serum albumin (HSA) may be utilized to form a sensor protein. During operation in a sensing device, glucose oxidase reacts with oxygen and oxidizes. The oxidation of glucose oxidase results in the formation of a hydroperoxy adduct, which transforms into hydrogen peroxide.

The applicant has recognized that, an obstacle to creating sensors that are long-lived and stable over time has been that glucose oxidase, when immobilized (e.g., for use in a sensor), undergoes oxidative inactivation by the aforementioned hydrogen peroxide over time. Since the lifetime of glucose sensors primarily depends on the lifetime of glucose oxidase, the effects of the peroxide on the glucose oxidase can severely limit the lifetimes of glucose sensors.

Prior processes for addressing the peroxide degradation of glucose oxidase have involved the use of additives or neutralizing agents for deactivating, removing or neutralizing peroxide. (Examples of such prior art are discussed below with respect to the Valdes et al. reference, the

Stemmer patent, the Hatzinikolaou et al. article, and the Wagner et al. patent). Embodiments of the present invention relate to an unexpected change in direction of the state of the art by employing directed evolution techniques to formulate a glucose oxidase gene having desired peroxide resistant properties.

Evolution under non-stress circumstances takes years. Accordingly, evolution may be manipulated in embodiments of the invention for specific enzymatic functions. In embodiments of the invention, a technique known as directed evolution is employed to evolve glucose oxidase, to formulate a glucose oxidase that possesses improved resistance to peroxide. A glucose oxidase formulated pursuant to embodiments of the present invention may improve the longevity of a biosensor in which it is employed.

According to the claims under appeal, a method comprises formulating an enzyme by obtaining an organism with a glucose oxidase gene. Using that organism growing multiple colonies of the organism. Next altering the environment of the colonies and screening the colonies to identify colonies with active glucose oxidase.

The multiple colonies are then screened for desirable peroxide resistant properties. The colonies are screened by determining whether the colonies contain active glucose oxidase and determining whether the colonies have desired peroxide resistant properties. Determining whether the colonies have desired peroxide resistant properties involves incubating the colonies in peroxide and determining whether the colonies have active glucose oxidase after incubating, including measuring a concentration of the glucose oxidase.

One embodiment of the invention involves, for example, a library of organisms, all of which contain glucose oxidase. In one embodiment, this library of organisms is grown in separate colonies with a conventional growth medium. In this embodiment, the environment of each colony is subsequently altered. For example, the environment of each colony may be altered by introducing peroxide to it. A screening procedure may be employed after the environments of the colonies have been altered. The screening procedure may involve processes

of determining which of the colonies contain active glucose oxidase. Those colonies that still contain active glucose oxidase after their environments have been altered may possess desirable peroxide resistant qualities. Glucose oxidase from those colonies still containing active glucose oxidase may be tested for functionality, for example, by immobilizing the glucose oxidase in a sensor. In other embodiments of the invention, following at least a portion of the screening procedure, the environments of the colonies may be altered another time if desired. For example, in one embodiment, altering the environments of the colonies by adding more peroxide may reduce the number of colonies that proceed to the functionality testing.

Those colonies that contain active glucose oxidase after the alteration of their environments and incubation procedures may possess desirable peroxide resistant qualities. Glucose oxidase from those colonies containing active glucose oxidase may be tested for functionality, for example, by immobilizing the glucose oxidase in a sensor. In other embodiments of the invention, following at least a portion of the screening procedure, the environments of the colonies may be altered another time if desired, for example, by adding more peroxide.

The method recited in the pending claims of the present application can provide significant advantages over the prior art of record. The ability to form a stable enzyme which is peroxide resistant and which may be employed in an altered environment (oxygen free environment), such as a biosensor, can provide significant advantages in extending the life of biosensors. When used in an implanted medical device (such as an implanted blood glucose sensor), peroxide resistance and, thus, a capability for extending the life of the enzyme can provide considerable patient comfort and safety advances, for example, by reducing the frequency of surgical sensor replacements. Moreover, the ability to form enzymes with peroxide resistant properties suitable for biosensor applications in a relatively inexpensive, non-complicated and reliable process can provide significant advantages with respect to the ability to manufacture readily available supplies of the enzyme and, thus, increasing the availability of longer-life biosensors to more patients.

By a method in accordance with embodiments of the present invention, a glucose oxidase enzyme may be formulated to exhibit desired peroxide resistant properties. As such, further additives or other mechanisms for deactivating, removing or neutralizing peroxide may not be required. Thus, the disclosed method involves a distinct departure from the conventional direction of those skilled in the art.

Claim 1	Specification
A method for formulating an enzyme comprising:	Title; pg. 1, ll. 21-24; pg. 4, ll. 10-22; and pg. 7, ll. 11-12.
obtaining an organism with a glucose oxidase gene;	Pg. 5, ll. 14-16; pg. 8, ll. 6-13; Fig. 2, ref. 12; pg. 14, ll. 1-8.
growing multiple colonies of the organism;	Pg. 5, l. 17-18; pg. 10, ll. 1-9; Fig. 2, ref. 18.
Altering the environment of the colonies	Pg. 6, l. 16 – 17; pg. 14 l. 9-10; Fig. 4, ref. 46.
screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies.	Pg. 5, ll. 18-21; pg. 10, l. 10 to pg. 13, l. 15; pg. 14, ll. 15-20; Fig. 2, ref. 20.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 1-17 are rejected, as follows:

1. Claims 1-3, 7-14, 17 and 19 are rejected under 35 U.S.C. 103(a) as being unpatenable over Valdes et al., Cherry et al. and Hatzinikolaou et al.

2. Claims 15-16 are rejected under 35 U.S.C. 103(a) as being unpatenable over Valdes et al., Cherry et al. and Hatzinikolaou et al. and further in view of MIXONIX

3. Claims 4-6 are rejected under 35 U.S.C. 103(a) as being unpatenable over Valdes et al., Cherry et al. and Hatzinikolaou et al. and further in view of Wagner and the Aldrich Catalog.

As noted in Section III, above, the present appeal relates to each of the above rejections and, thus, all of the rejected claims (i.e., claims 1-17 and 19).

VII. ARGUMENT

1. Appeal Of Rejection Of Claims 1-3, 7-14, 17 and 19 Under 35 U.S.C. § 103(a).

Claims 1-3, 7-14 and 17 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Valdes et al., Cherry et al. and Hatzinikolaou et al. This rejection is respectfully traversed in view of the following remarks.

Claim 1 recites a method for formulating an enzyme that is not disclosed by either Valdes et al., Cherry et al., or Hatzinikolaou et al., alone or in the combination proposed by the Examiner (which combination is respectfully traversed as discussed herein). For example, the method of claim 1 recites, among other features:

"obtaining an organism with a glucose oxidase gene; growing multiple colonies of the organism; altering the environment of the colonies; and screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies."

Claim 1 recites several actions that, together, form the claimed method, where not any one or a combination of the above-cited references describes the combination of actions recited in claim 1. The cited references fail to teach, suggest or render predictable selecting pieces of the processes in Valdes et al., Cherry et al. and Hatzinikolaou et al. and combining them in the fashion that the Examiner suggests. Instead, the references, themselves, as well as other references of record teach a direction away from the present invention.

The mass of evidence of record in the application suggests that those skilled in the art were taking a direction that was completely different from that of the claimed invention. While the Examiner raises arguments as to obviousness to combine various parts of the cited references, none of the evidence of record supports the Examiner's proposal to select and combine portions of the various references. To the contrary, a number of references of record (including the primary reference relied upon by the Examiner) teach a direction different than the claimed invention and would lead one of ordinary skill in the art away from the claimed invention. Without the present disclosure as a guide, one of ordinary skill in the art would not have found it obvious to combine the above-cited references as suggested by the Examiner.

As described in more detail below:

a. The prior art of record does not teach or suggest or render predictable the claimed invention;

b. None of the prior art of record provide any teaching or suggestion or render predictable the combination of the Valdes et al, Cherry et al., and Hatzinikolaou et al., as proposed by the Examiner, and the mass of evidence of record shows that the prior art teaches away from the claimed invention; and

c. Each of dependent claims 2-3 and 7-8 recites further features that distinguish those claims from the prior art. Accordingly, those claims do not stand or fall with claim 1.

a. The Rejection Is Improper Because The Prior Art Does Not Teach Or Suggest Or Render Predictable The Claimed Invention.

In particular, neither Valdes et al. nor Cherry et al., nor Hatzinikolaou et al. describe formulating a glucose oxidase enzyme by growing multiple colonies of a organism, altering the environment of the colonies, and screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies. Moreover, one of ordinary skill in the art would not have been led by the prior art of record to alter the environment of such colonies,

much less screen for active glucose oxidase. Such procedures would have been a drastic departure from the state of the art and, without the benefit of the present specification as a guide, would not have been obvious to one of ordinary skill in the art.

The Examiner argues that Valdes et al. teaches that glucose oxidase in glucose sensors degrade over time due to hydrogen peroxide. The Examiner acknowledges that Valdes et al. do not teach a method of producing mutant glucose oxidase that is resistant to degradation from peroxide. (Office Action of August 7, 2008, pg. 4, ll. 12-14.) As discussed in more detail below, instead, Valdes et al. teach addressing peroxide degradation by adding a chemical catalase or by attaching an immobilized enzyme to a support that deactivates hydrogen peroxide. (Valdes, pg. 375, Left Column, ll. 6-18.

The Examiner refers to Valdes et al.'s statement than to ensure longer sensor functionality, instead of replacing a degraded glucose oxidase sensor enzyme with a fresh enzyme, it is advantageous to "prevent the degradation of the enzyme" (Office Action of August 7, 2008, pg. 4, ll. 3-6, citing Valdes et al., pg. 375, ll. 2-5). The Examiner attempts to use that statement out of context, as a springboard to imply that Valdes et al. would have suggested a process involving altering the environment of the colonies of glucose oxidase organism and screening colonies in the manner recited in the present claims. However, Valdes et al. immediately follow the above statement with a description of the use of chemical additives as the so-called "better options." Accordingly, Valdes et al. teach a specific direction (use of chemical additives) that departs from the then-conventional process of replacing a degraded enzyme with a fresh enzyme.

Valdes et al. is not the only reference of record that teaches that the direction taken by those skilled in the art was to use chemical additives. Indeed, other references of record similarly teach that direction of the art (e.g., U.S. Patent No. 6,689,265 to Heller et al. and the article titled "Glucose ENFET doped with MnO_2 powder" by Yin et al., Evidence Appendix Exhibits 1 and 2). Neither Valdes et al., nor any prior art of record that relates to peroxide degradation of glucose oxidase, describe or suggest altering the environment of the glucose oxidase colonies and

screening the colonies for peroxide resistant properties for addressing peroxide degradation of glucose oxidase. Instead, as described in more detail below, Valdes et al. and other references of record show that the direction taken by those skilled in the art was away from the method of the presently claimed invention.

Because of this lack of disclosure in Valdes et al., the Examiner attempts to select pieces of each of the Cherry et al. and the Hatzinikolaou et al. references. However, neither of those references teach formulating a glucose oxidase enzyme by altering the environment of the glucose oxidase colonies to make them resistant to peroxide degradation.

For example, as noted above, claim 1 recites a method for formulating an enzyme that includes, among other features, "obtaining an organism with a glucose oxidase gene" and "growing multiple colonies of the organism." In addition, claim 1 recited "alterning the environment of the colonies" and "screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies." The Examiner stated that Cherry et al. disclose a method of making mutants of an enzyme which is also degraded in the presence of hydrogen peroxide, by using directed evolution techniques. (Office Action of August 7, 2008, pg. 4, ll. 18-21.) In addition, the Examiner stated:

> "Cherry et al. discloses that after multiple rounds of directed evolution an enzyme, mutants of said enzyme that are resistant to deactivation in the presence of high concentration of hydrogen peroxide, conditions that mimic of hydrogen peroxide wherein the enzyme is normally deactivated, were obtained (pages 380-382). Cherry et al. discloses that colonies having enzymatic activity were selected to determine for its resistance against hydrogen peroxide (page 382)." (Office Action of August 7, 2008, pg. 4, l. 21 to pg. 5, l. 4.)

However, Cherry et al., like Valdes et al., fail to disclose or suggest "obtaining an organism with glucose oxidase genes" and "growing multiple colonies of the organism." As such, Cherry et al. also fail to disclose or suggest "altering the environment" of such colonies or

"screening" such colonies for active glucose oxidase after altering the environment of the colonies. Cherry et al. have nothing to do with glucose oxidase genes and would not teach or suggest growing colonies of an organism with glucose oxidase, or screening such colonies for active glucose oxidase. Instead, Cherry et al. describe production of a detergent additive (having no glucose oxidase) that is able to catalyze the oxidation of dyes that leach out of colored clothing during a wash cycle to render the dyes colorless and effectively prevent the transfer of dye to other clothes. (Cherry, et al., pg. 379, col. 1, ll. 24-29.)

According to Cherry, et al., "[i]n wash conditions using bleach-containing detergents, the elevated pH and high peroxide concentrations favor rapid formation of inactive form of the enzyme." Cherry et al. state that the "goal" of their work "was to develop a peroxidase variant effective as a dye-bleaching reagent in detergent." (Cherry et al., pg. 379, col. 2, ll. 7-11.) Cherry et al. teach to produce a dye-bleaching reagent in a clothes washing detergent and do not teach or suggest anything to do with obtaining an organism with a glucose oxidase gene and growing multiple colonies of the organism, much less screening such colonies for active glucose oxidase after altering the environment of the colonies

The Examiner's apparent attempt to rely on Cherry et al. as teaching of directed evolution of enzymes, in general, to be resistant to peroxide takes Cherry et al.'s disclosure far out of context. **The only reason that Cherry et al. is concerned about peroxide resistance is because Cherry et al.'s stated goal was to develop a dye-bleaching reagent in a clothes washing detergent (where "wash conditions" have "high peroxide concentrations").** Cherry et al.'s "inactivation conditions were designed to mimic those found in washing machines using bleach containing commercial detergents," at pH levels and temperatures ("40-50 C") inconsistent with the production of glucose oxidase. (Cherry et al., pg. 380, col. 1, ll. 19-21.) There is no logical relation between Cherry et al.'s clothes washing detergent (or the high peroxide environment of a clothes wash cycle) and glucose oxidase enzymes for glucose sensors. Cherry et al.'s reference to peroxide resistance and inactivation conditions for a dye bleaching reagent in a wash cycle would not teach or suggest anything to one skilled in the art about

glucose oxidase, much less to obtain an organism with a glucose oxidase gene, grow multiple colonies of the organism and/or screen such colonies for active glucose oxidase after altering the environment of the colonies.

The context in which Cherry et al. refer to peroxide resistance (clothes washing environments) would not have been ignored by one of ordinary skill in the art. Such contexts (and environments) are inconsistent with the production of glucose oxidase. Thus, like Valdes et al., Cherry et al. fail to disclose or suggest the invention recited in claim 1.

The Examiner stated that Hatzinikolaou et al. discloses a library of glucose oxidase genes known in the art. (Office Action of August 7, 2008, pg. 5, ll. 5-6.) However, Hatzinikolaou et al. describe isolating and characterizing a new synthesized glucose oxidase for purposes of conducting certain specified analyses (described on pages 373 and 374 of the Hatzinikolaou et al. reference), none of which relate to resistance to hydrogen peroxide (claim 3), or altering the environment of the glucose oxidase organism colonies and thereafter screening the colonies for active glucose oxidase (claim 1).

While gene libraries have been employed by those skilled in the art for gene analysis, Hatzinikolaou et al. provide no suggestion to use such libraries in the formulation of an enzyme by directed evolution. Hatzinikolaou et al. teaching of using gene libraries to analyze characteristics of a gene provides no motivation or suggestion or render predictable to do anything more than to analyze the specific new synthesized glucose oxidase for the specific characteristics described on pages 373 and 374 of that reference. The Examiner has picked only the feature of forming a gene library of glucose oxidase gene from Hatzinikolaou et al.'s overall process and seeks to combine that teaching with Valdes et al. and Cherry et al.

However, Hatzinikolaou et al.'s purpose of forming a library of a new simulated glucose oxidase (for analyzing the characteristics of the new simulated glucose oxidase described in that reference) would have no applicable purpose in any mutation process described by Cherry et al. Once Hatzinikolaou et al. obtains and isolates a sample of the new glucose oxidase,

Hatzinikolaou et al. conducts analysis on the isolated sample. Mutating the sample would not allow Hatzinikolaou et al. to analyze the characteristics of the simulated glucose oxidase (as the mutations could effect the detection of characteristics under analysis). Accordingly, it would not have been obvious to look to Hatzinikolaou et al. as a teaching of growing multiple colonies of an organism with a glucose oxidase, altering the environment to the colonies and screening the colonies to identify colonies with peroxide resistant properties. The Examiner's suggestion to combine Hatzinikolaou et al. with Cherry et al. is, therefore, respectfully traversed. Moreover, Hatzinikolaou et al. does not disclose or suggest screening colonies for active glucose oxidase after altering the environment of the colonies.

Because Cherry et al. do not relate to growing multiple colonies of an organism with a glucose oxidase gene or altering the environment of such colonies, it follows that Cherry et al. also do not describe screening such colonies by determining whether the colonies contain active glucose oxidase. Also, Hatzinikolaou et al. provide no teaching or suggestion of growing such colonies with a glucose oxidase gene and screening such colonies for active glucose oxidase after altering the environment of such colonies. While the combination of those references is traversed for reasons noted above, no combination of those references could lead to growing such colonies and screening the colonies for active glucose oxidase, because neither of those references, describe such features. Moreover, as noted above, Valdes et al. also do not teach screening colonies for active glucose oxidase after altering the environment of the colonies and, instead, teach a very different direction (addition of chemicals to reduce peroxide degradation). Accordingly, the Examiner's suggestion to combine Valdes et al. with Cherry et al. and Hatzinikolaou et al. is traversed and would not lead to the present invention.

None of the cited references describes or suggests creating colonies of an organism with a glucose oxidase gene, altering the environment of the colonies and then screening colonies for active glucose oxidase. Accordingly, the combination of the references (as suggested by the Examiner) could not result in the claimed invention. The rejection of claims 1-3, 7-14 and 17 under 35 U.S.C. 103(a) is, therefore, respectfully traversed and should be reversed.

b. The Rejection Is Improper Because Prior Art Provides No Motivation To Combine And Teaches Away From The Combination Suggested By The Examiner.

Because of the above-noted lack of disclosure in Valdes et al. (of growing multiple colonies of the glucose oxidase organism and screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies), the Examiner attempts to select pieces of each of the Cherry et al. and Hatzinikolaou et al. references, and combine those with the Valdes et al. reference.

None of the Valdes et al, Cherry et al. nor Hatzinikolaou et al. references provide any teaching of creating multiple colonies of glucose oxidase containing organism, altering the environment of the colonies and screening colonies for active glucose oxidase. Rather, Valdes et al. teach away from such methods by, instead, referring to **conventional procedures that use additives for deactivating or destroying hydrogen peroxide** and, thus, teach away from such a method, as follows:

"To prohibit the H_2O_2 from degrading the GOD enzyme, it has been proposed that catalase be coimmobilized with GOD... The addition of catalase in either the GOD itself, or to the incubating solution has resulted in a slower deactivation of the GOD enzyme ... A long term remedy of the degradation of GOD by H_2O_2 could be the immobilization and attachment of the enzyme to a support that deactivates H_2O_2, as it is being produced. Such as study was conducted by Cho[2], using the peroxide decomposition catalyst, activated carbon. In a study conducted by Carter[19], the best results were obtained with activated carbon, impregnated with ruthenium. This combination was able to destroy hydrogen peroxide and stabilized the enzyme." (Valdes et al., pg. 375, col. 1, l.18 to col. 2, l. 6.)

Not only does Valdes et al. fail to teach or suggest to alter the environment of glucose oxidase colonies or to screen the colonies to identify colonies with active glucose oxidase, but, in the above-quoted statement, Valdes et al. further teaches to use other, very different procedures (conventional in the art) to address degradation effects of peroxide on glucose oxidase. Thus, the

Valdes et al. reference shows that the direction taken by those most skilled in the art involved employing materials, additives, or the like that deactivate peroxide.

Additional art of record also describes conventional "additive" processes for removing or neutralizing peroxide such as by adding an antioxidant or peroxidase to the glucose oxidase to break down peroxide or by coating the glucose oxidase enzyme with a protective coating, including U.S. Patent No. 6,689,265 to Heller et al. and the article titled "Glucose ENFET doped with MnO_2 powder" by Yin et al. Those references further emphasize that, prior to the present invention, the direction taken by those skilled in the art for addressing the peroxide degradation of glucose oxidase was wholly different from the direction of the present invention. In U.S. Patent No. 6,689,265 to Heller et al., a peroxide generating enzyme may include a sufficiently thick, natural, electrically insulating protein or glycoprotein layer. (See column 6, lines 59-67 of the Heller et al. patent) Heller et al. also disclose an alternative embodiment in which a peroxide generating enzyme is immobilized in a non-conducting inorganic or organic polymeric matrix. (See column 7, lines 3-11 of the Heller et al. patent, Evidence Appendix Exhibit 2). Also, Heller et al. describe a first layer enzyme 11 (peroxidase) that reduces peroxide generated from a second layer (glucose oxidase layer) 13. The Yin et al. article describes the addition of MnO_2 to catalyze peroxide and produce water and oxygen therefrom. (Yin, Evidence Appendix Exhibit 2, Abstract and pg. 188, col. 1, ll. 20-34.)

Thus, both the Heller et al. patent and the Yin et al. article show that the direction taken by those skilled in the art is to provide additives or complex multi-layer sensor structures to remove hydrogen peroxide. These references, in addition to Valdes et al.'s express references to conventional uses of additives, show that those skilled in the art were not considering growing, altering and screening colonies for peroxide resistance glucose oxidase organism, but instead were attempting to address the peroxide production issue by removing or neutralizing peroxide with additives (not by altering the glucose oxidase). The state and direction of the industry, as evidenced by Valdes et al., Heller et al. and Yin et al., was a wholly different direction than that taken by the present Applicants (including altering the environment of the glucose oxidase

colonies, screen the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies (claim 1). Accordingly, the mass of evidence of record (including the primary reference relied upon by the Examiner) teaches one skilled in the art taking a direction different from (and away from) the present invention.

The mass of evidence of record showing the direction of the industry (away from that of the present invention) cannot be ignored. Without the present disclosure as a guide, one of ordinary skill in the art would not have found Valdes et al.'s discussion of the degradation of glucose oxidase as a prompt or suggestion to employ a mutation process for detergent as described Cherry et al. Instead, as noted above, one of ordinary skill in the art would have looked to conventional manners of removing peroxide, such as additives for removing or neutralizing peroxide. Accordingly, the rejection of 1-3 and 7-8 under 35 U.S.C. 103(a) is further respectfully traversed.

The fact that the primary reference (Valdes et al.) teach away from the clamed invention and the combination suggested by the Examiner, shows that a *prima facie* case of obviousness has not been raised. Numerous Federal Circuit decisions recognize that an invention will not be deemed obvious in a patent law sense when one or more prior art references "teach away" from the invention. For example, the Federal Circuit stated "as a useful general rule, that references that teach away cannot serve to create a prima facie case of obviousness." *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1354, 60 USPQ2d 1001 (Fed. Cir. 2001). Last April, *KSR Int'l Co. v. Teleflex Inc*, the U.S. Supreme Court again acknowledged that principle, by citing its previous decision in *Untied States v. Adams*, 383, U.S. 39, 40 (1966), in which the Court relied upon a principle that when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious. The U.S. Supreme Court further stated "[a]s is clear from cases such as *Adams*, a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently known in the prior art." (*KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1734, 82 USPQ2d 1385, 1391 (2007).)

Furthermore, "an applicant may rebut a *prima facie* case of obviousness by showing that the prior art teaches away from the claimed invention in any material respect." *In re Peterson*, 315 F.3d 1325, 1331, 65 USPQ2d 1379 (Fed. Cir. 2003). Also see, *Gillette Co. v. S.C. Johnson & Sons, Inc.*, 919 F.2d 720, 724, 16 USPQ2d 1923, 1927 (Fed. Cir. 1990) (the closest prior art reference "would likely discourage the art worker from attempting the substitution suggested by [the inventor/patentee]") and *Singh v. Brake*, 317 F.3d 1334, 1346, 65 USPQ2d 1641 (Fed. Cir. 2003)("whether or not a reference 'teaches away' from a claimed invention" is "relevant in determining whether or not a claimed invention would have been obvious").

When patentability turns on the question of obviousness, the search for and analysis of the prior art includes evidence relevant to the finding of whether there is a teaching, motivation, or suggestion to select and combine the references relied on as evidence of obviousness. (underline added for emphasis.) *See, e.g., McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1351—52, 60 USPQ2d 1001, 1008 (Fed. Cir. 2001) ("the central question is whether there is reason to combine [the] references," a question of fact drawing on the *Graham* factors).

Conclusive statements that prior art references provide motivation to combine, or statements of motivation derived from the Applicant's own specification, are not sufficient to set forth a *prima facie* case of obviousness. "The factual inquiry whether to combine references must be thorough and searching." *Id.* It must be based on objective evidence of record. This precedent has been reinforced in myriad decisions. *See, e.g., Brown & Williamson Tobacco Corp. v. Philip Morris Inc.*, 229 F.3d 1120, 1124— 25, 56 USPQ2d 1456, 1459 (Fed. Cir. 2000) ("a showing of a suggestion, teaching, or motivation to combine the prior art references is an 'essential component of an obviousness holding'") (quoting *C.R. Bard. Inc., v. M3* Systems, *Inc.*, 157 F.3d 1340, 1352, 48 USPQ2d 1225, 1232 (Fed. Cir. 1998)); *In re Dembiczak,* 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999) ("Our case law makes clear that the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references."); *In re Dance,* 160 F.3d 1339, 1343, 48 USPQ2d 1635, 1637 (Fed. Cir. 1998) (there

must be some motivation, suggestion, or teaching of the desirability of making the specific combination that was made by the applicant); *In re Fine,* 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988) ("'teachings of references can be combined *only* if there is some suggestion or incentive to do so.') (emphasis in original) (quoting *ACS Hosp.* Sys., *Inc. 'v. Montefiore Hosp.,* 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984)).

As noted above, the Examiner has not shown any motivation or suggestion in the prior art that would have led one skilled in the art to grow colonies of an organism with a glucose oxidase gene, alter the environment of those colonies and screen for active glucose oxidase after altering the environment. In fact, Valdes et al and other prior art of record show that altering the environment and screening process for glucose oxidase would have been a drastic diversion from the direction taken by those most skilled in the prior art when seeking to address peroxide degradation of glucose oxidase.

The legal authority expresses the requirement for a showing of specificity in the prior art of motivation to select components to combine. *See, e.g., In re Kotzab,* 217 F.3d 1365, 1371, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000) ("particular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed"); *In re Rouffet,* 149 F.3d 1350, 1359, 47 USPQ2d 1453, 1459 (Fed. Cir. 1998) ("even when the level of skill in the art is high, the Board must identify specifically the principle, known to one of ordinary skill, that suggests the claimed combination. In other words, the Board must explain the reasons one of ordinary skill in the art would have been motivated to select the references and to combine them to render the claimed invention obvious."); *In re Fritch,* 972 F.2d 1260, 1265, 23 USPQ2d 1780, 1783 (Fed. Cir. 1992) (the examiner can satisfy the burden of showing obviousness of the combination "only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references").

While the Valdes et al., Cherry et al. and Hatzinikolaou et al. references, themselves, provide no motivation or suggestion to combine teachings, the Examiner argues that one of ordinary skill in the art would have been motivated to do so to formulate or produce mutant glucose oxidase that is resistant to peroxide (Office Action of August 7, 2008, pg. 5, ll. 10-13). While this is the likely conclusion, after reading the present disclosure as a guide, the references of record actually teach to do something very different (add chemicals) to reduce peroxide degradation of glucose oxidase. Thus, the mass of evidence of record shows that the motivation provided by the cited references would have been to reduce peroxide degradation by adding chemicals as taught by Valdes et al., U.S. Patent No. 6,689,265 to Heller et al. and the article by Yin et al.

The Examiner further argues that "[o]ne of ordinary skill in the art would have been motivated to produce mutant peroxide resistant glucose oxidases in order to use them in glucose sensors, thereby prolonging their use, since Valdes et al. teaches that glucose oxidases in glucose sensors are degraded by peroxide." (Office Action of August 7, 2008, pg. 5, ll. 19-22.) However, as noted above, one of ordinary skill in the art would not have ignored Valdes et al.'s (and others) express teaching of using additives to address peroxide degradation and, thus, would have been taught in a direction away from producing mutations of glucose oxidase to address peroxide resistance. The record shows that the cited references (and Valdes et al., in particular) would have motivated those skilled in the art in a direction away from the present claims.

In addition, the Examiner argues that one of ordinary skill in the art would have had a "reasonable expectation of success." However, without the present disclosure as a guide, one of ordinary skill in the art would not have selected altering the environment of glucose oxidase organism colonies, screening the colonies, purifying, isolating and measuring processes to modify Valdes et al.'s disclosed solution to peroxide degradation of glucose oxidase. Valdes et al. teaches solutions to the peroxide degradation problem (by using chemical additives) and would have led one skilled in the art in the direction of those solutions.

Cherry et al. do not mention glucose oxidase anywhere in their disclosure. Moreover, the only relevance that Cherry et al. has to peroxide is in the context of a clothes washing detergent and a clothes washing environment (pH and temperature) that would not be compatible with the production of glucose oxidase for biological sensors. Thus, Cherry et al. does not address the deficiency of Valdes et al.

Hatzinikolaou et al. fails to provide any motivation or suggest any relation to altering the environment of the glucose oxidase organism colonies or of addressing peroxide degradation of glucose oxidase. Moreover, the whole purpose of Hatzinikolaou et al. (to analyze a specific new simulated glucose oxidase) is not consistent with Cherry et al.'s mutation process.

The Examiner's conclusive statements of suggestion to combine, and the Examiner's argument of "reasonable expectation of success," fail to address the significant issue of why one skilled in the art would have been motivated to select a process as described by Cherry et al., to change the direction taken by those most skilled in the prior art as described by Valdes et al. The Examiner's argument that a "reasonable expectation of success" would have motivated the combination, is contrary to the express teachings of the prior art. The prior art teaches that those most skilled in the art were taking a wholly different direction to address peroxide degradation of glucose oxidase and, thus, would have found it unreasonable (not reasonable) to change the course of direction from that of the state of the art.

More specifically, Valdes et al. refer to completely different directions taken by those most skilled in the art, whereby the glucose oxidase enzyme is immobilized and attached to a support that deactivates peroxide. "A reference may be said to teach away when a person of ordinary skill, upon reading the reference,... would be led in a direction divergent from the path that was taken by the applicant." *Tec Air, Inc. v. Denso Mfg. Mich. Inc.*, 192 F.3d 1353, 1360, 52 USPQ2d 1294, 1298 (Fed. Cir. 1999). Valdes et al., directly refers the reader to conventional methods of addressing peroxide degradation of glucose oxidase that employ additives for destroying or neutralizing peroxide (which is quite different from creating multiple colonies, altering the environment and screening for desired peroxide resistant properties).

Because the Examiner has not shown any motivation or suggestion in the prior art that would have led one skilled in the art to select Cherry et al.'s mutation process and materially change the direction taught by the Valdes et al. reference, the Examiner has not raised a *prima facie* case of obviousness. Therefore, the rejection of 1-3, 7 and 8 under 35 U.S.C. 103(a) is respectfully traversed.

c. Each of dependent claims 2, 3, 7-14 and 17 recite further features that distinguish those claims from the prior art.

Each of dependent claims 2, 3, 7-14 and 17 recite further featured that distinguish those claims from the prior art. In particular, each of those claims recites features relating to altering the environment of the colonies and screening the colonies to identify colonies with active glucose oxidase. As described above, neither the Valdes et al., Cherry et al., nor Hatzinikolaou et al. references describe or suggest or render predictable altering the environment of the colonies and screening the colonies for active glucose oxidase after altering the environment. In that regard those references also do no disclose or suggest the additional processing recited in dependent claims 2, 3, 7-14 and 17, including:

1. "the organism is selected from a group consisting of *Aspergillus Niger, Penecillium funiculosum, Saccharomyes cervisiae, and Escherichia Coli*" (claim 2);

2. "altering the environment of the colonies comprises introducing peroxide to the colonies" (claim 3);

3. "testing the colonies with active glucose oxidase for a predefined, desired functionality after screening the colonies to identify colonies with active glucose oxidase" (claim 7); and

4. "continuing to alter the environments of the colonies until the colonies with active glucose oxidase are of a suitable number to proceed with testing the colonies with active glucose oxidase for the predefined, desired functionality" (claim 8).

5. "testing the colonies with active glucose oxidase for the predefined, desired functionality comprises employing glucose oxidase from the colonies in sensors" (claim 9);

6. "testing the colonies further comprises: extracting glucose oxidase from the colonies; immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies; placing the immobilized glucose oxidase in a sensor; and testing the sensor" (claim 10);

7. "extracting glucose oxidase from the colonies comprises employing an ionic column to extract glucose oxidase from the colonies" (claim 11);

8. "extracting glucose oxidase from the colonies comprises: removing the glucose oxidase from the colonies; purifying the glucose oxidase; and characterizing the glucose oxidase" (claim 12);

9. "removing the glucose oxidase from the colonies comprises grinding the colonies in a homogenizer into cell components" (claim 13);

10. "removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer" (claim 14);

11. "purifying the glucose oxidase comprises purifying the glucose oxidase by employing chromatography methods" (claim 17).

12. "testing the sensor comprises introducing the sensor into a test environment and testing the sensor for satisfactory sensing functionality" (claim 19)

The rejection of claims 2, 3, 7-14, 17 and 19 is, therefore respectfully traversed and should be reversed.

2. Appeal Of Rejection Of Claims 15 And 16 Under 35 U.S.C. 103(a)

Claims 15 and 16 are rejected under 35 U.S.C. 103 (a) as being unpatentable over Valdes et al., Cherry et al., and Hatzinikolau et al. as applied to claims 1-3, 7-14 and 17 above, and further in view of MISONIX.

This rejection is respectfully traversed at least for reason discussed above with respect to claim 1. Each of claims 15 and 16 is indirectly dependent on claim 1. Accordingly, the distinctions noted above between claim 1 and the cited Valdes et al., Cherry et al. and Hatzinikolaou et al. references apply to claims 15 and 16, as well. The MISONIX reference was not relied upon by the Examiner to address those distinctions. Instead, the Examiner cited the MISONIX reference as allegedly teaching of disrupting cells via sonication.

Accordingly, at least for reasons discussed above with respect to claim 1, the rejection of claims 15 and 16 is respectfully traversed.

Furthermore, while the Examiner stated that "[o]ne of ordinary skill in the art would have been motivated to [combine the teachings of Valdes et al., Charry et al. and Hatzinikolaou et al. with MISONIX] in order to disrupt cells comprising mutant glucose oxidase" and "would have had a reasonable expectation of success since disruption of cells using sonication is well known and practiced routinely in the art." (Office Action dated August 7, 2008, pg. 12, ll. 12-15.) However, MISONIX does not provide any motivation or suggestion of disrupting glucose oxidase cells in a process as recited in claims 15 and 16, where glucose oxidase is extracted from colonies that have been screened for active glucose oxidase by disrupting cell components via sonication (claim 15) and fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication (claim 16).

The Examiner's comments do not address the features of fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies with sonication, as recited in claim 16. Accordingly, the Examiner has failed to provide a prima facie

case of obviousness of claim 15 or of claim 16. The rejection of claim 15 and 16 is traversed and should be reversed.

3. Appeal of Rejection Of Claims 4-6 Under 35 U.S.C. 103(a)

Claims 4-6 are rejected under 35 U.S.C. 103 (a) as being unpatentable over Valdes et al., Cherry et al., and Hatzinikolau et al. as applied to claims 1-3, 7-14 and 17 above, and further in view of Wagner and Aldrich Catalog.

This rejection is respectfully traversed at least for reason discussed above with respect to claim 1. Each of claims 4-6 is directly or indirectly dependent on claim 1. Accordingly, the distinctions noted above between claim 1 and the cited Valdes et al., Cherry et al. and Hatzinikolaou et al. references apply to claims 4-6, as well. The Wagner reference was not relied upon by the Examiner to address those distinctions and does not address the above-noted distinctions between the claims and the Valdes et al., Cherry et al. and Hatzinikolaou references.

Indeed, the Wagner reference was cited, according to the Examiner, for disclosing a method of determining glucose oxidase activity via a sensor by measuring fluorescence emission from a dye, wherein oxidation of glucose by active glucose oxidase reduces the fluorescence emission. However, Wagner does not teach or suggest formulating a glucose oxidase enzyme by growing colonies, altering the environment of the colonies and screening the colonies for active glucose oxidase after altering the environment. Accordingly, the combination of Wagner with the above-discussed references (the Valdes et al., Cherry et al. and Hatzinikolaou references) would not lead to the presently claimed invention.

The Examiner also cited the Aldrich Catalog as describing Leuco-crystal violet dyes as common fluorescent dyes. The cited portion of the Aldrich Catalog neither describes nor suggests formulating an enzyme, much less growing colonies, altering the environment of the colonies and screening the colonies for active glucose oxidase after altering the environment.. Accordingly, the cited portion of the Aldrich Catalog does not address the above-noted distinctions between the claimed invention and the Valdes et al., Cherry et al., Hatzinikolaou et

al. and Wagner references. Thus, the combination of the cited portion of the Aldrich Catalog with those other references (as suggested by the Examiner) could not result in the claimed invention.

Accordingly, the rejection of claims 4-6 should be reversed.

Conclusion

In view of the foregoing, it is respectfully submitted that claims 1-17 and 19 are in condition for allowance and the application should be allowed in its present form. In particular, it is respectfully submitted that the presently pending rejections of claims 1-17 and 19 are improper and should be reversed for reasons as discussed above. In that regard, each of claims 1-17 and 19 is in condition for allowance.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741.

If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date: November 26, 2008

By: _____

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VIII. CLAIMS APPENDIX

1. (Original) A method for formulating an enzyme comprising:

obtaining an organism with a glucose oxidase gene;

growing multiple colonies of the organism;

altering the environment of the colonies; and

screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies.

2. (Previously Presented) A method for formulating an enzyme according to claim 1, wherein the organism is selected from a group consisting of *Aspergillus Niger, Penecillium funiculosum, Saccharomyces cerevisiae,* and *Escherichia Coli.*

3. (Original) A method for formulating an enzyme according to claim 1, wherein altering the environment of the colonies comprises introducing peroxide to the colonies.

4. (Original) A method for formulating an enzyme according to claim 1, wherein screening the colonies to identify colonies with active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

5. (Original) A method for formulating an enzyme according to claim 4, wherein the substance is leuco-crystal-violet.

6. (Previously Presented) A method for formulating an enzyme according to claim 1, wherein screening the colonies to identify colonies with active glucose oxidase comprises checking for fluorescence.

7. (Previously Presented) A method for formulating an enzyme according to claim 1, wherein the method further comprises testing the colonies with active glucose oxidase for a predefined, desired functionality after screening the colonies to identify colonies with active glucose oxidase.

8. (Previously Presented) A method for formulating an enzyme according to claim 7, wherein the method further comprises continuing to alter the environments of the colonies until the colonies with active glucose oxidase are of a suitable number to proceed with testing the colonies with active glucose oxidase for the predefined, desired functionality.

9. (Previously Presented) A method for formulating an enzyme according to claim 7, wherein testing the colonies with active glucose oxidase for the predefined, desired functionality comprises employing glucose oxidase from the colonies in sensors.

10. (Previously Presented) A method for formulating an enzyme according to claim 7, wherein testing the colonies with active glucose oxidase for the predefined, desired functionality comprises:

extracting glucose oxidase from the colonies;

immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies;

placing the immobilized glucose oxidase in a sensor; and

testing the sensor.

11. (Original) A method for formulating an enzyme according to claim 10, wherein extracting glucose oxidase from the colonies comprises employing an ionic column to extract glucose oxidase from the colonies.

12. (Original) A method for formulating an enzyme according to claim 10, wherein extracting glucose oxidase from the colonies comprises:

removing the glucose oxidase from the colonies;

purifying the glucose oxidase; and

characterizing the glucose oxidase.

13. (Original) A method for formulating an enzyme according to claim 12, wherein removing the glucose oxidase from the colonies comprises grinding the colonies in a homogenizer into cell components.

14. (Original) A method for formulating an enzyme according to claim 13, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer.

15. (Original) A method for formulating an enzyme according to claim 12, wherein removing the glucose oxidase from the colonies comprises disrupting the colonies into cell components via sonication.

16. (Original) A method for formulating an enzyme according to claim 15, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication.

17. (Original) A method for formulating an enzyme according to claim 12, wherein purifying the glucose oxidase comprises purifying the glucose oxidase by employing chromatography methods.

18. (Withdrawn) An enzyme formulated according to the method of claim 1.

19. (Previously Presented) A method for formulating an enzyme according to claim 10, wherein testing the sensor comprises introducing the sensor into a test environment and testing the sensor for satisfactory sensing functionality.

IX. EVIDENCE APPENDIX

Exhibit 1: U.S. Patent No. 6,689,265 to Heller et al.

Exhibit 2: Article enttitled "Glucose ENFET doped with MnO_2 powder" by Yin et al.

X. RELATED PROCEEDINGS APPENDIX

None.

EXHIBIT 1

US006689265B2

(12) **United States Patent**
Heller et al.

(10) **Patent No.: US 6,689,265 B2**
(45) **Date of Patent: *Feb. 10, 2004**

(54) **ELECTROCHEMICAL ANALYTE SENSORS USING THERMOSTABLE SOYBEAN PEROXIDASE**

(75) Inventors: **Adam Heller**, Austin, TX (US); **Gregg L. Kenausis**, Austin, TX (US); **Qiang Chen**, Austin, TX (US); **Mark S. Vreeke**, Alameda, CA (US)

(73) Assignee: **TheraSense, Inc.**, Alameda, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 131 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/815,747**

(22) Filed: **Mar. 23, 2001**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. 09/291,230, filed on Apr. 13, 1999, now abandoned, which is a continuation of application No. 08/798,596, filed on Feb. 11, 1997, now Pat. No. 5,972,199, which is a continuation-in-part of application No. 08/540,789, filed on Oct. 11, 1995, now Pat. No. 5,665,222.

(51) **Int. Cl.**[7] **G01N 27/327**

(52) **U.S. Cl.** **204/403.09**; 204/403.04; 204/403.14

(58) **Field of Search** 204/403.04, 403.09, 204/403.1, 403.11, 403.14

(56) **References Cited**

U.S. PATENT DOCUMENTS

3,781,212 A 12/1973 Smillie
3,796,634 A 3/1974 Haynes et al.

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

EP 0 390 390 A1 10/1990
EP 0 439 318 A2 7/1991

(List continued on next page.)

OTHER PUBLICATIONS

User's Manual for YSI 2300 Stat Plus Glucose & L-Lactate Analyzer, Yellow Springs Instruments, Inc. Yellow Springs, Ohio, believed available before Oct. 1995.*
Liu et al., "Reagentless amperometric biosensors highly sensitive . . . mediator between horseradish peroxidase and an electrode," Analytica Chimica Acta 344 (1997) 187–199.*

(List continued on next page.)

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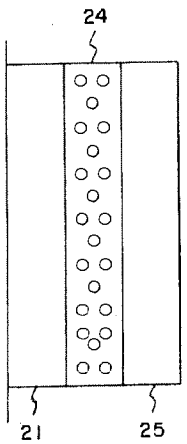
(57) **ABSTRACT**

A sensor for the detection and measurement of an analyte in a biofluid. The sensor includes two enzymes. One type of sensor measures the concentration of hydrogen peroxide using a thermostable peroxidase enzyme that is immobilized in a redox hydrogel to form a sensing layer on a working electrode. This sensor also includes a hydrogen peroxide-generating second enzyme which is insulated from the redox hydrogel and electrode. This second enzyme generates hydrogen peroxide in response to the presence of an analyte or analyte-generated compound. The second enzyme may be insulated from the electrode by placement of an electrically insulating layer between the sensing layer and the second enzyme layer.

Alternatively, the second enzyme is immobilized in an inorganic polymeric matrix, preferably made using a sol-gel polymerization process. Such matrices include those made of silica. Often, the second enzyme is stabilized by immobilization in a sol-gel. Further stabilization of polyelectrolytic enzymes can be obtained by immobilizing the enzyme with a polyelectrolytic polymer in the sol-gel matrix.

20 Claims, 20 Drawing Sheets



U.S. PATENT DOCUMENTS

3,911,901	A		10/1975	Niedrach et al.
4,102,746	A		7/1978	Goldberg
4,458,686	A		7/1984	Clark, Jr.
4,781,798	A		11/1988	Gough
4,840,893	A		6/1989	Hill et al.
4,938,860	A		7/1990	Wogoman
5,165,407	A		11/1992	Wilson et al.
5,240,843	A		8/1993	Gibson et al.
5,264,092	A		11/1993	Skotheim et al.
5,278,046	A		1/1994	Johnson et al.
5,292,801	A		3/1994	Avnir et al.
5,300,564	A		4/1994	Avnir et al.
5,308,495	A		5/1994	Avnir et al.
5,320,725	A		6/1994	Gregg et al.
5,334,296	A		8/1994	Henkens et al.
5,352,348	A		10/1994	Young et al.
5,643,721	A		7/1997	Spring et al.
5,665,222	A	*	9/1997	Heller et al. 205/792
5,964,993	A		10/1999	Blubaugh, Jr.
5,972,199	A	*	10/1999	Heller et al. 205/777.5

FOREIGN PATENT DOCUMENTS

JP	62155099	7/1987
JP	02087056	3/1990
JP	02268684	11/1990
RU	2 049 991	12/1995
WO	WO 93/20230	10/1993
WO	WO 93/23748	11/1993
WO	WO 95/10605	4/1995
WO	WO 97/13870	4/1997

OTHER PUBLICATIONS

Liu et al., "Entrapment of both glucose oxidase and peroxidase in regenerated silk fibroin membrane," Fresenius J. Anal. Chem. (1996) 355, 78–82.*

Chuang et al., "Purification and Characterization of Vegetable Soybean Peroxidase (II). CHaracterization of Vegetable Soybean Peroxidase Isoenzymes," Shih P'in K'o Hsueh (Taipei) (1988), 15(1), 12–26.*

Aharonson et al., Recent Developments in Organically Doped Sol–Gel Sensors: A Microns–Scale Probe: Successful Trapping of Purified Polyclonal Antibodies; Solutions to the Dopant–Leaching Problem, in "Better Ceramics Through Chemistry", Sanchez et al. Eds. Materials Res. Soc. Symp. Proc., 346:1–12 (1994).

Alvarez–Icaza, et al., Mass Production of Biosensors, Anal. Chem., 65:525A–533A (1993) Jun. 1, 1993.

Asther et al., Immobilization as a Tool for the Stabilization of Lignin Peroxidase Produced by Phanerochaete chrysosporium INA–12, Applied Biochemisry and Biotechnology, 38:57–67 (1992).

Audebert, et al., Electrochemical Probing of the Activity of Glucose Oxidase Embedded Sol–Gel Matrices, Chem. Mater, 5:911–913 (1993).

Avnir, et al., Enzymes and Other Proteins Entraped in Sol–Gel Materials, Chem. Mater., 6:1605–1614 (1994).

Avnir, Organic Chemistry within Ceramic Matrices: Doped Sol–Gel Materials, Acc. Chem. Res., 28:328–334 (1995).

Braun, et al., Biochemically active sol–gel glasses; the trapping of enzymes, Materials Letters, 10:1–5 (1990).

Chien, et al., An immobilized–enzyme system for the determination of sialic acid using cloned neuraminidase from Clostridium perfringens A.T.C.C. 10543, Biotechnol. Appl. Biochem., 19:51–60 (1994).

Csoregi et al., Design, Characterization and One–point in Vivo Calibration of a Subcutaneously Implanted Glucose Electrode, *Anal. Chem.,* 66, 3131–3138 (1994).

Dave et al., Synthesis of Protein–Doped Sol–Gel SiO2 Thin Films: Evidence for Rotational Mobility of Encapsulated Cytochrome, Chem. Mater., 7:1431–1434 (1995).

Ellerby, et al., Encapsulation of Proteins in Transparent Porous Silicate Glasses Prepared by the Sol–Gel Method, Science, 255:1113–1115 (1992).

Enzymol International, Inc. 2543 Westbelt Dr., Columbus, OH 43228, Product Literature Oct. 1995.

Fukushima, et al., A New Immobilization Technique of Whole Cells and Enzymes with Colloidal silica and Alginate, Biotechnology and Bioengineering, 32:584–594 (1988).

Gibson et al, Stabilization of Analytical Enzymes Using a Novel Polymer–Carbohydrate System and the Production of a Stabilized, Single Reagent for Alcohol Analysis, Analyst, 117:1293–1297 (1992).

Gibson et al., Extended shelf life of enzyme–based biosensors using a novel stabilization system, Biosensors & Bioelectronics, 7:701–708 (1992).

Gibson et al., Preservation of shelf life of enzyme based analytical systems using a combination of sugars, sugar alcohols and cationic polymers of zinc ions, Analytica Chimic Acta, 279:185–192 (1993).

Gibson et al., The Stabilization of Analytical Enzymes Using Polyelectrolytes and Sugar Derivatives, in "Stability and Stabilization of Enzymes", van den Tweel et al. Eds., Elsevier Science Publishers B.V. (1993).

Gorton et al., Selective detection in flow analysis based on the combination of immobilized enzymes and chemically modified electrodes, Analytica Chimica Acta, 250:203–248 (1991).

Gregg, et al., Redox Polymer Films Containing Enzymes. 1. A Redox–Conducting Epoxy Cement: Synthesis, Characterization, and Electrocatalytic Oxidation of Hydroquinone, J. Phys. Chem., 95:5970–5975 (1991).

Hatayama, et al., Immobilizaiton of Urease on Composite Fibre by Using a Gel Formation of Cellulose Acetate and Titanium Iso–Propxide, Journal of Sol–Gel Science and Technology 7:13–17 (1996).

Haynes, et al., Enzyme Envelopes on Colloidal Particles, Biochemical and Biophysical Research Communications, 36:235–242 (1969).

Kallury, et al., Enhanced Stability of Urease Immobilized onto Phospholipid Covelently Bound to Silica Tungsten, and Fluoropolymer Surfaces, Anal. Chem., 65:2459–2467 (1993) Sep. 15, 1993.

Kallury, et al., Enhancement of the Thermal and Storage Stability of Urease by Covalent Attachment to Phospholipid–Bound Silica, Anal. Chem., 64:1062–1068 (1992) May 1, 1992.

Katakis, et al., Electrostatic Control of the Electron Transfer Enabling Binding of Recombinant Glucose Oxidase and Redox Polyelectrolytes, J. Am. Chem. Soc., 116:3617–3618 (1994).

Katsube et al., "Stabilization of an FET Glucose Sensor with the Thermophilic Enzyme Glucokinase", *Sens. Actuators,* B (1990), B1(1–6), 504–7.

Kenausis, G., "Electrochemical Gluclose and Lactate Sensors Based on "Wired" Thermostable Soybean Peroxidase Operating Continuously and Stabley at 37° C.", *Anal. Chem.,* 69(6):1054–1060 (Mar. 15, 1997).

Kulys, et al., Mediatorless peroxidase electrode and preparation of bienzyme sensors, Bioelectrochemistry and Bioenergetics, 24:305–311 (1994).
Marcinkeviciene, J. et al., "Bienzyme Strip–type Glucose Sensor", *Biosensors & Bioelectronics,* 8:209–212 (1993).
McNeil, et al., Thermostable Reduced Nicotinamide Adenine Dinucleotide Oxidase: Application To Amperometric Enzyme Assay, Anal. Chem., 61:25–29 (1989).
Paddock, et al., Electrocatalytic reduction of hydrogen peroxide via direct electron transfer from pyrolytic graphite electrodes to irreversibly absorbed cytochrome c peroxidase, J. Electoanal. Chem., 260:487–494 (1989).
Pankratov et al., Sol–gel derived renewable–surface biosensors, Journal of Electroanalytical Chemistry, 393:35–41 (1995).
Ryan, et al., Thermostabilized chemical derivatives of horseradish peroxidase, Enzyme Microb. Technol., 16:501–505 (1994).
Sampath, et al. Sol–Gel Derived Ceramic–Carbon Enzyme Electrodes: Glucose Oxidase as a Test Case, Journal of Sol–Gel Science and Technology, 7:123–128 (1996).
Shtelzer et al., An optical biosensor based upon glucose oxidase immobilized in sol–gel silicate matrix, Biotechnol. Appl. Biochem., 19:293–305 (1994).
Suekane, Immoblization of glucose isomerase, Zeitschrift fur Allgemeine Mikrobiologie, 22:565–576 (1982).
Tatsuma, et al., Enzyme Monolayer and Bilayer–Modified Tin Oxide Electrodes for the Determination of Hydrogen Peroxide and Glucose, Anal. Chem., 61:2352–2355 (1989).
Vreeke, et al., Hydrogen Peroxide and β–Nicotinamide Adenine Dinucleotide Sensing Amperometric Electrodes Based on Electrical Connection of Horseradish Peroxidase Redox Centers to Electrodes through a Three–Dimensional Electron Relaying Polymer Network, Anal. Chem., 64:3084–3090 (1992).
Vreeke, et al., Hydrogen Peroxide Electrodes Based on Electrical connection of Redox Centers of Various Peroxidases to Electrodes through a Three–Dimensional Electro–Relaying Polymer Network, in "Diagnostic Biosensor Polymers" ACS Symposium Series 556:180–193 (1994).
Vreeke, M.S. et al., "A Thermostable Hydrogen Peroxide Sensor Based on "Wiring" of Soybean Peroxidase", *Anal. Chem.,* 67(23):4247–4249 (Dec. 1, 1995).
Wang et al., Screen–Printable Sol–Gel Enzyme–Containing Carbon Inks, Anal. Chem., 68:2705–2708 (1996).
Wang et al., Sol–Gel–Derived Metal–Dispersed Carbon Composite Amperometric Biosensors, Electroanalysis, 9:52–55 (1997).
Wang, et al., Amperometric biosensing of organic peroxides with peroxidase–modified electrodes, Anal. Chim. Acta, 254:81–88 (1991).
Weetall, Storage Stability of Water–Insoluble Enzymes Covalently Coupled to Organic and Inorganic Carriers, Biochim. Biophys. Acta. 212:1–7 (1970).
Yamanaka, et al., Enzymatic Activity of Glucose Oxidase Encapsulated in Transparent Glass by the Sol–Gel Method, Chem. Mater, 4:495–497 (1992) May/Jun.
Yang, et al., Applications of "Wired" Peroxidase Electrodes for Peroxide Determination in Liquid Chromatography Coupled to Oxidase Immobilized Enzyme Reactors, Anal. Chem., 67:1326–1331 (1995).
Ye et al., Influence of additives on the thermostability of glucose oxidase, Enzyme Microb. Technol., 10:498–502 1988).
Zaitseva, et al., Stabilization of heterogeneous amylose catalysts, Russian Journal of Physical Chemistry 66:1662–1664 (1992).
Zusman et al., Doped Sol–Gel Glasses as Chemical Sensors, Journal of Non–Crystalline Solids, 122:107–109 (1990).

* cited by examiner

FIG. 1

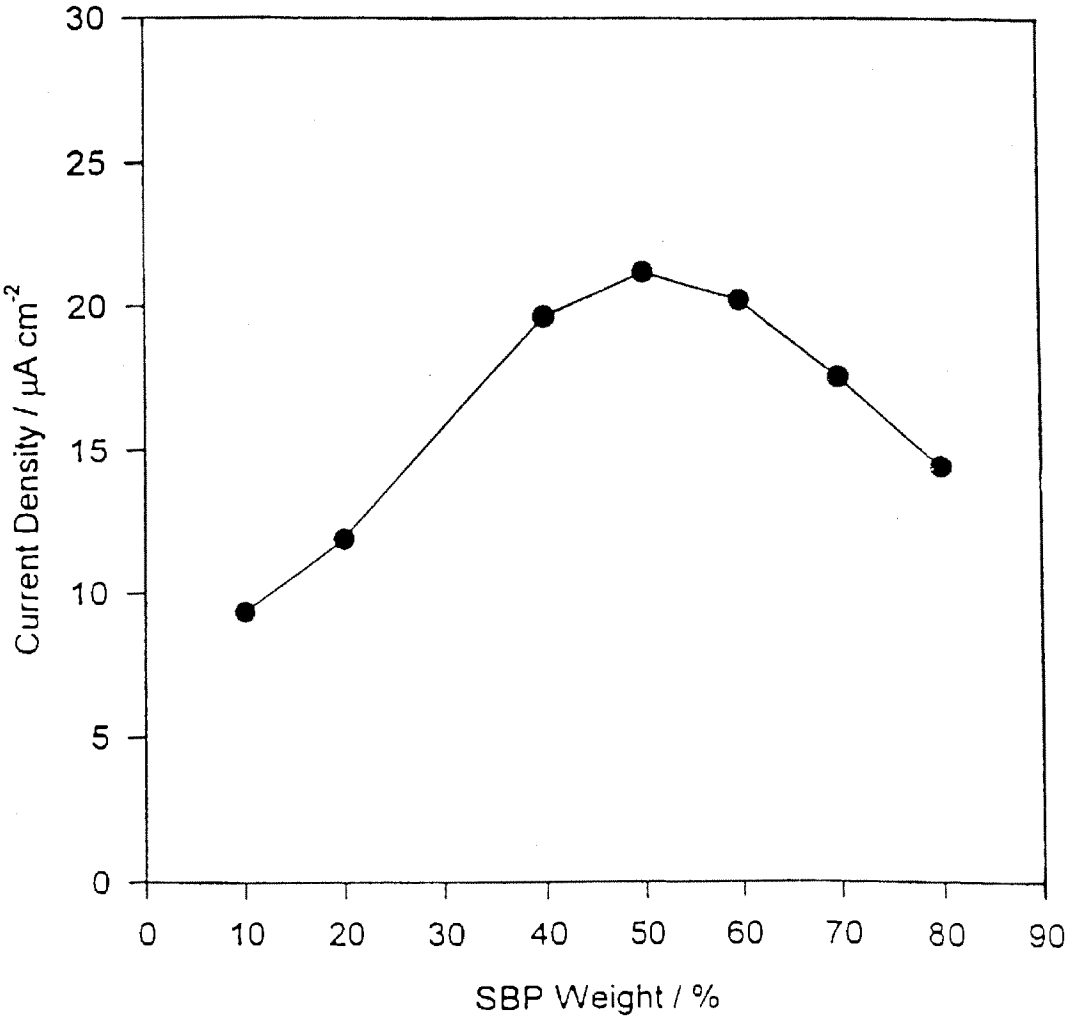

30
25
20
15
10
5
0
Current Density / μA cm-2
0
10
20
30
40
50
60
70
80
90
SBP Weight / %

FIG. 2

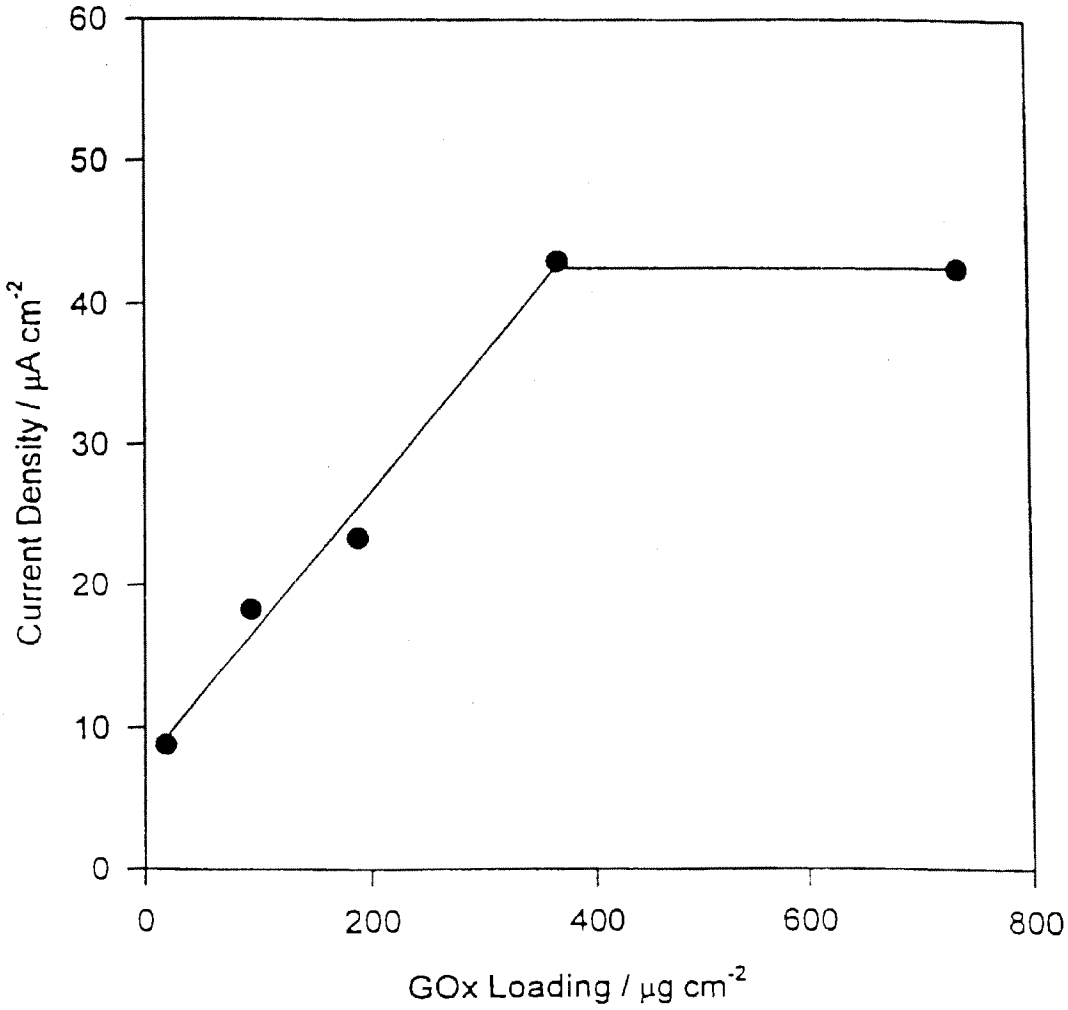

60
50
40
30
20
10
0
Current Density / μA cm-2
0
200
400
600
800
GOx Loading / μg cm-2

FIG. 3

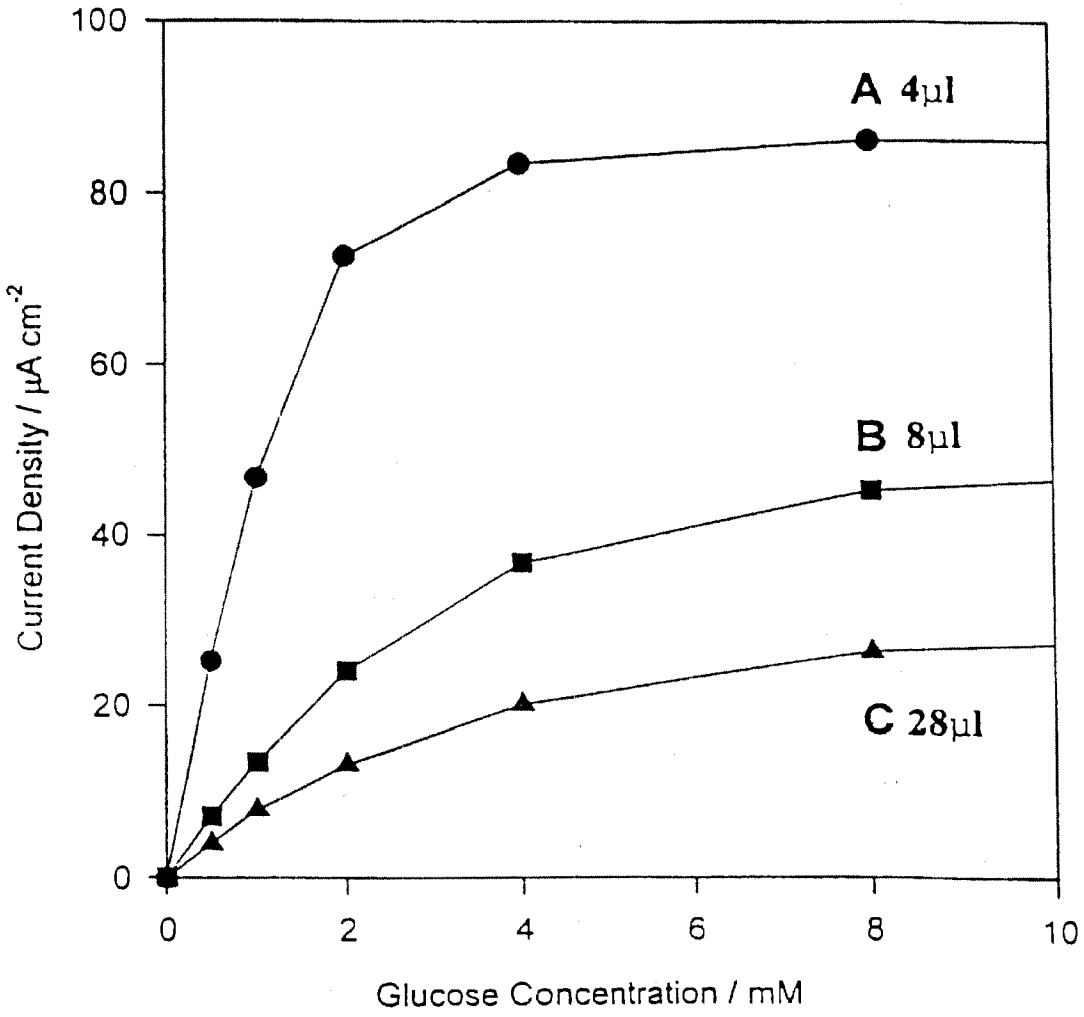

100
80
60
40
20
0
Current Density / μA cm-2
A 4μl
B 8μl
C 28μl
0
2
4
6
8
10
Glucose Concentration / mM

FIG. 4

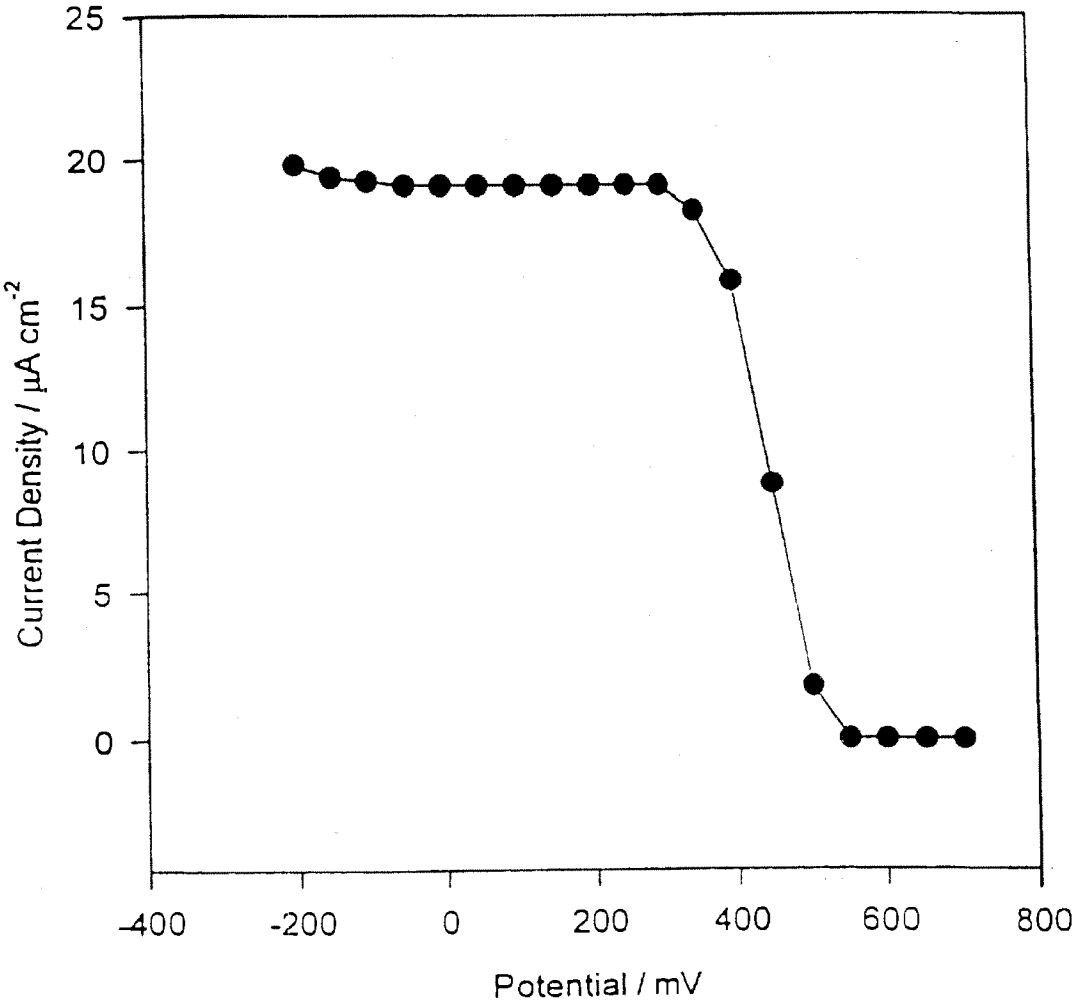

25
20
15
10
5
0
Current Density / μA cm-2
-400
-200
0
200
400
600
800
Potential / mV

FIG. 5

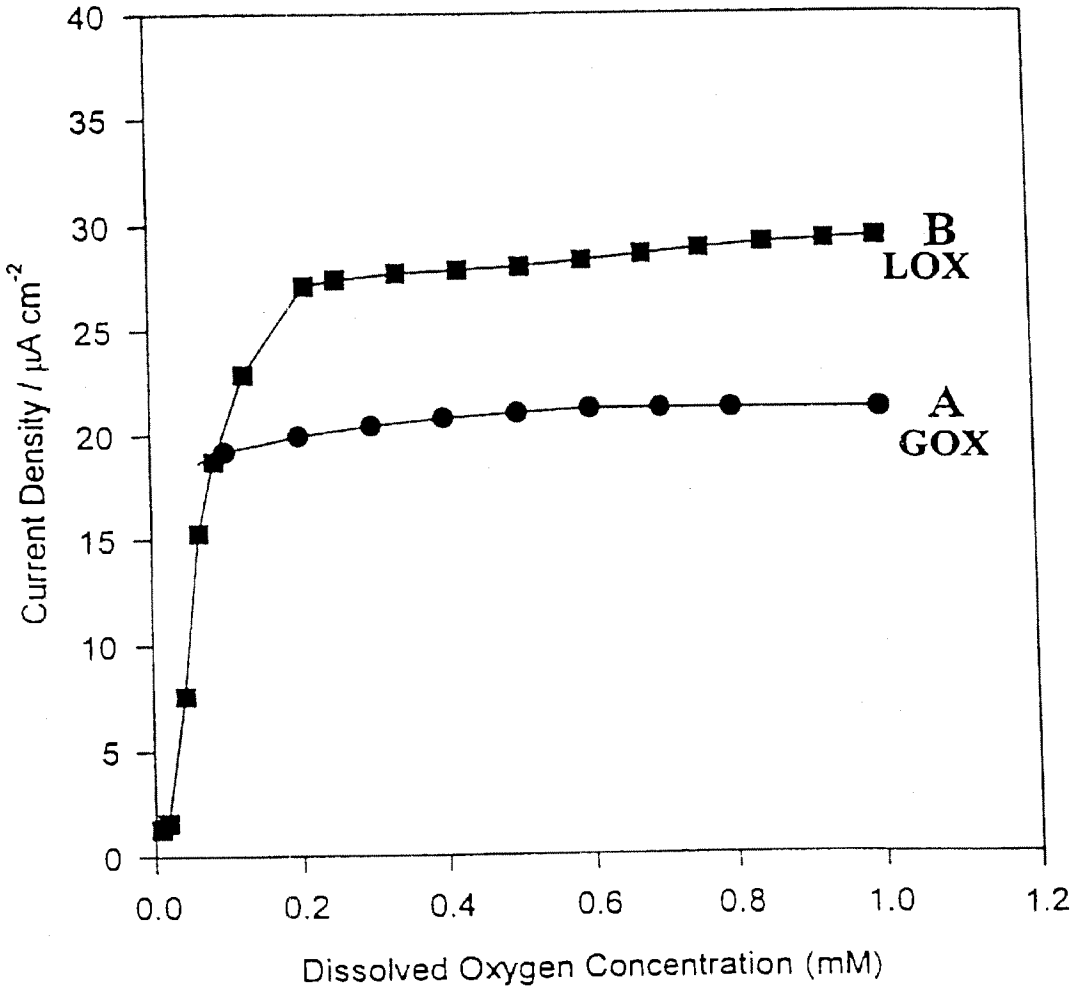
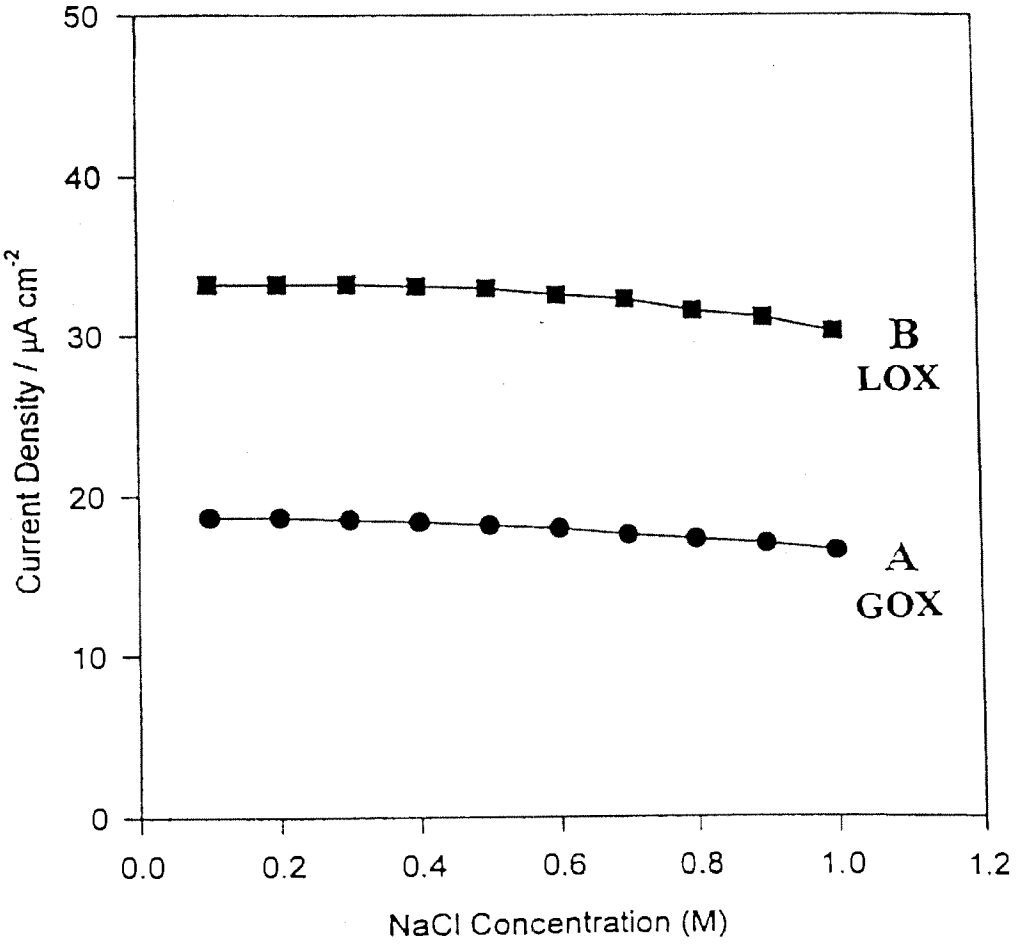

40
35
30
25
20
15
10
5
0
Current Density / μA cm-2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Dissolved Oxygen Concentration (mM)
B
LOX
A
GOX

FIG. 6


50
40
30
20
10
0
Current Density / μA cm-2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
NaCl Concentration (M)
B
LOX
A
GOX

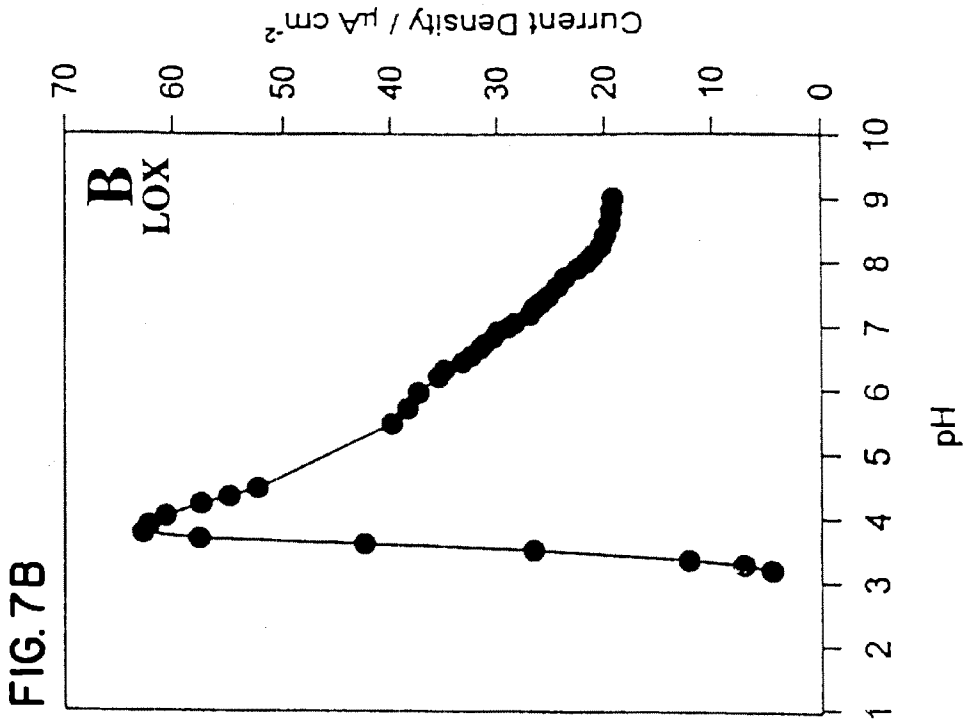

B
LOX
Current Density / μA cm-2
0
10
20
30
40
50
60
70
1 2 3 4 5 6 7 8 9 10
pH

FIG. 7B

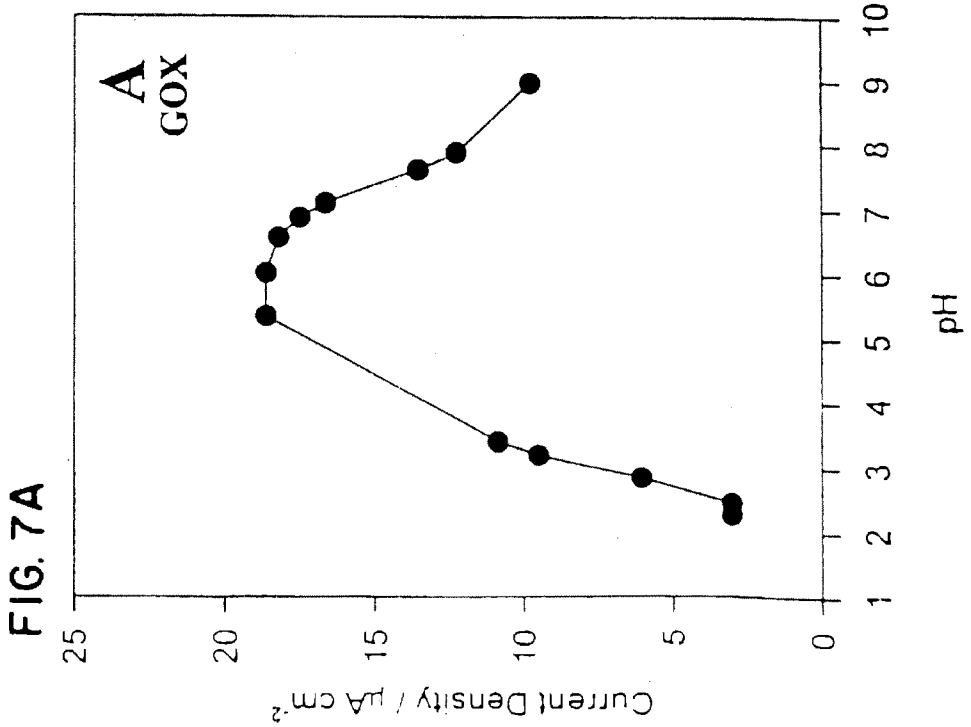
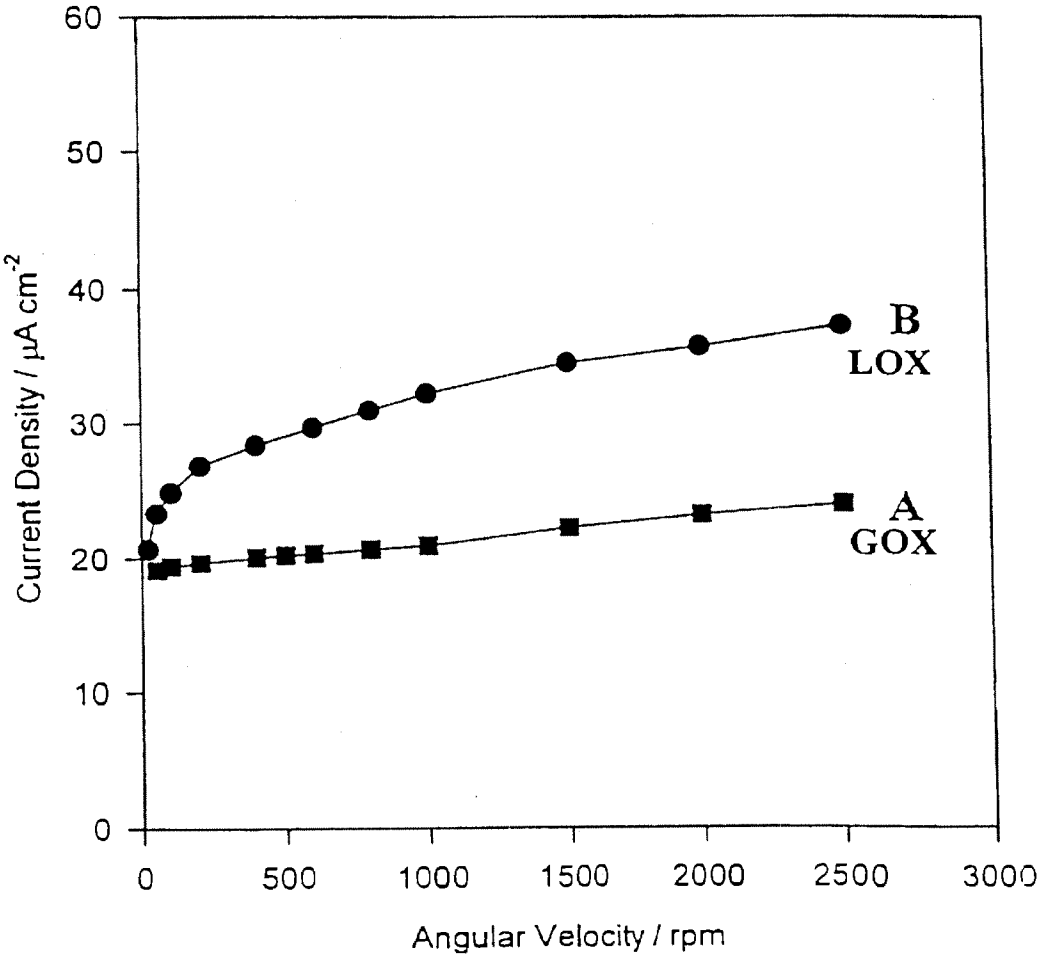

A
GOX
Current Density / μA cm-2
0
5
10
15
20
25
1 2 3 4 5 6 7 8 9 10
pH

FIG. 7A

FIG. 8


60
50
40
30
20
10
0
Current Density / μA cm-2
0
500
1000
1500
2000
2500
3000
Angular Velocity / rpm
B
LOX
A
GOX

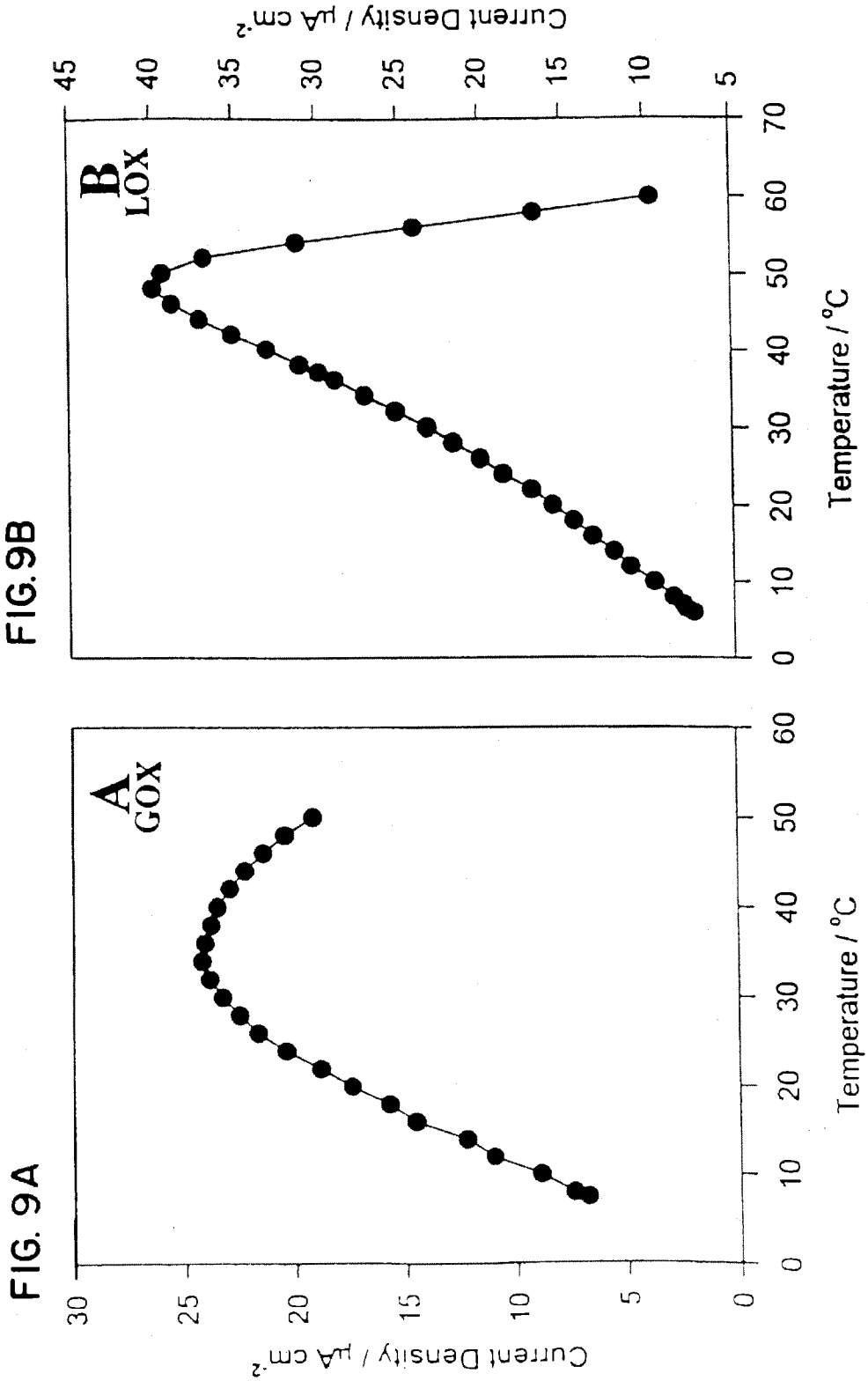

A
GOX
B
LOX
Current Density / μA cm-2
Temperature / °C
0
10
20
30
40
50
60
70
5
15
25
35
45

FIG. 9A

FIG.9B

FIG. 10A

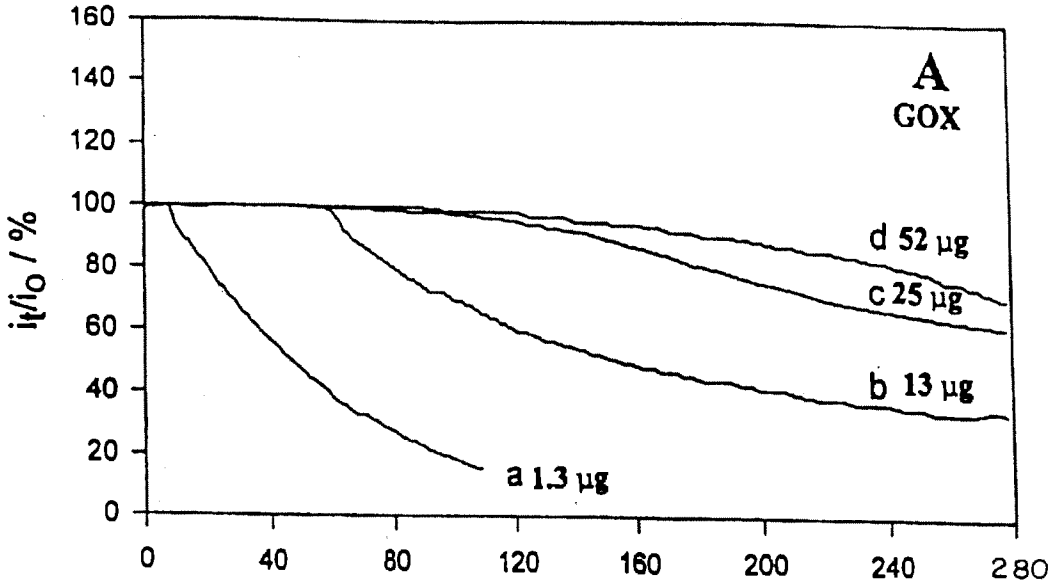


FIG. 10B

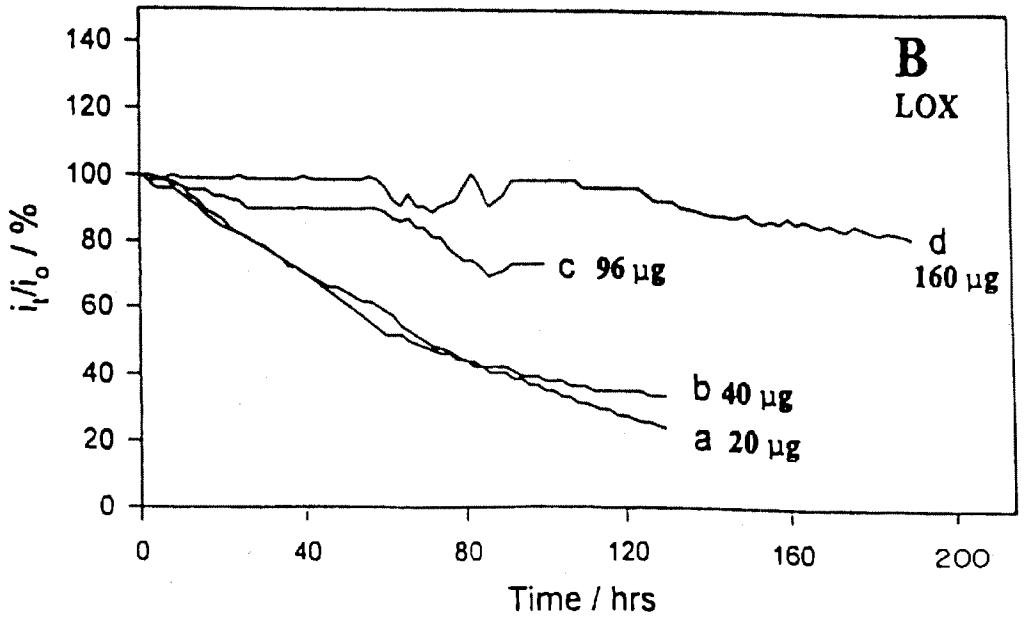


FIG. 11

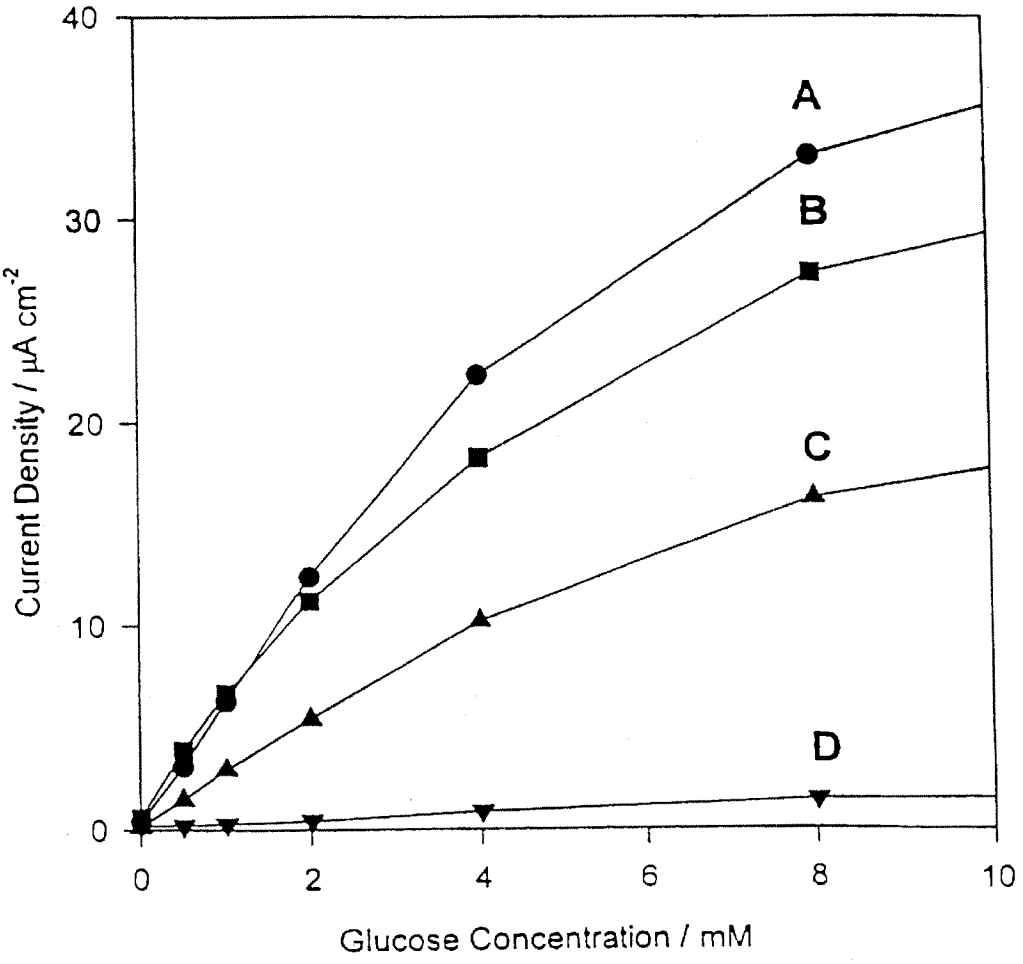

40
30
20
10
0
Current Density / μA cm-2
0
2
4
6
8
10
Glucose Concentration / mM
A
B
C
D

FIG. 12

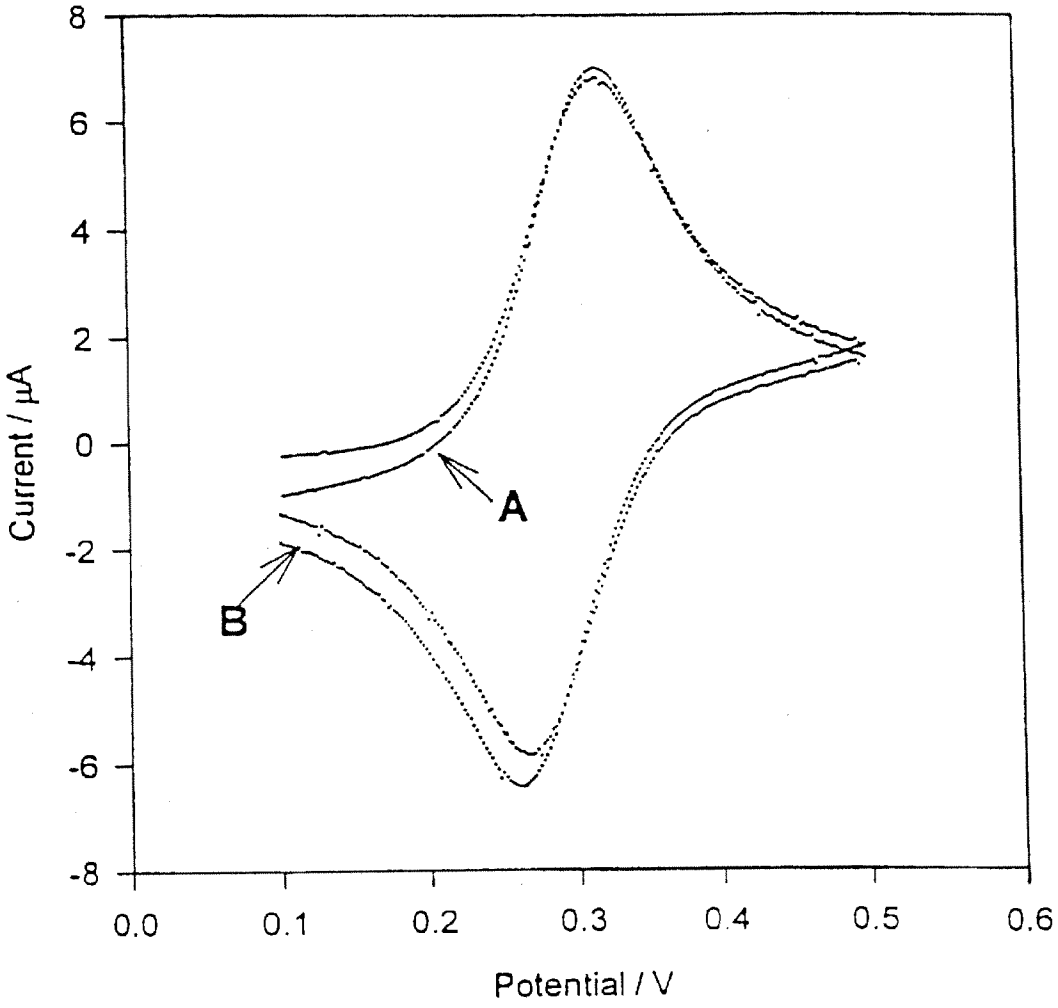
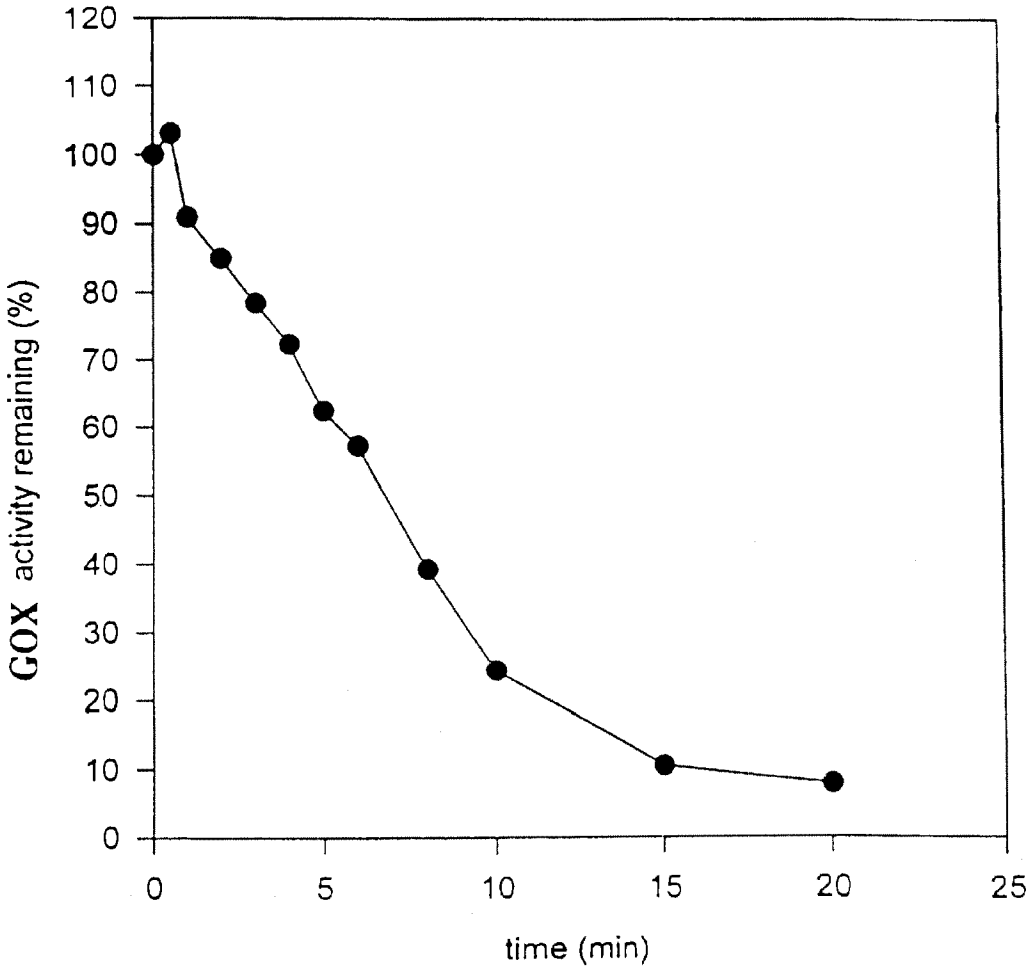


FIG. 13


120
110
100
90
80
70
60
50
40
30
20
10
0
GOX activity remaining (%)
0
5
10
15
20
25
time (min)

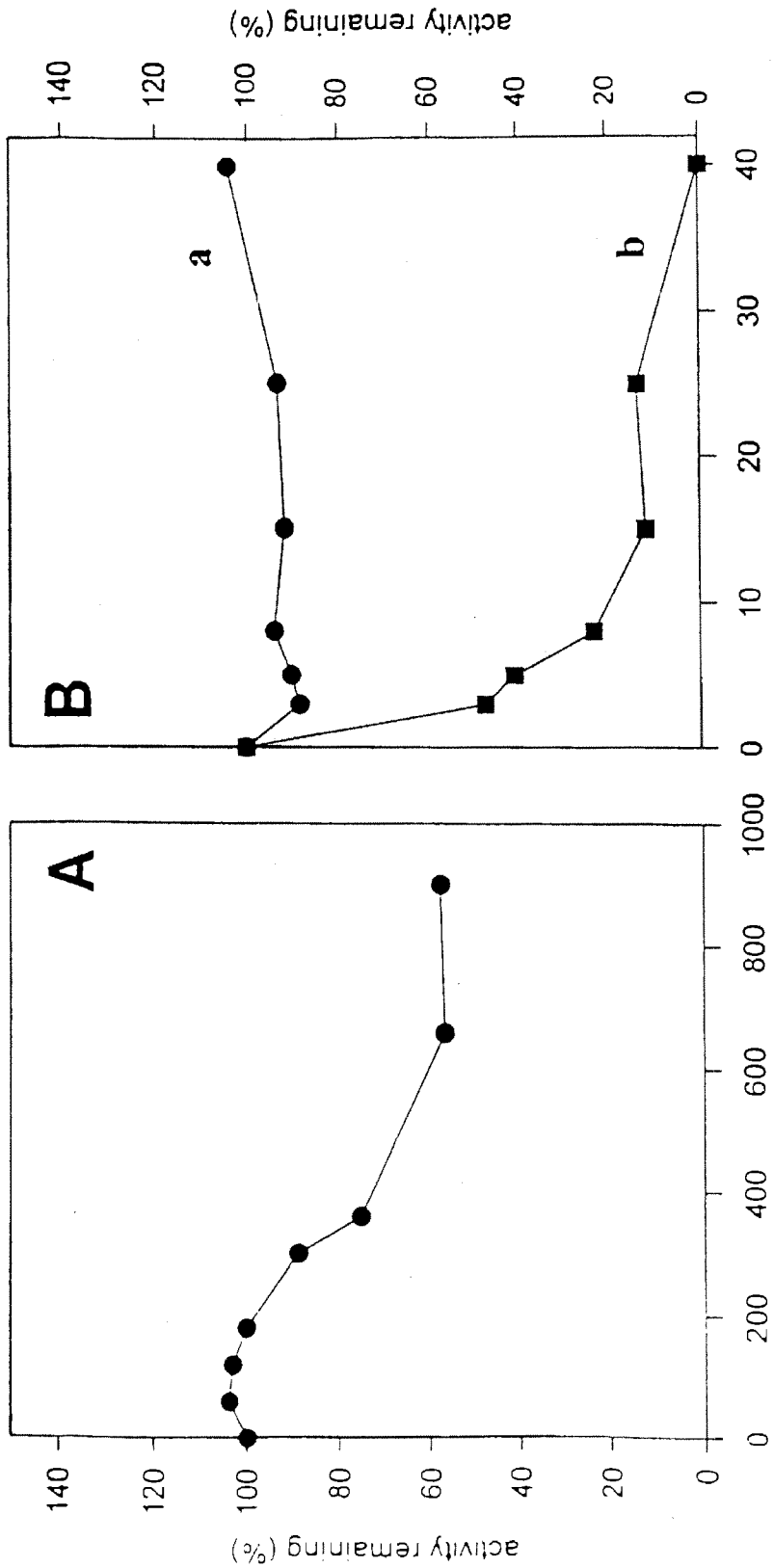


FIG. 14A

FIG. 14B

FIG. 15

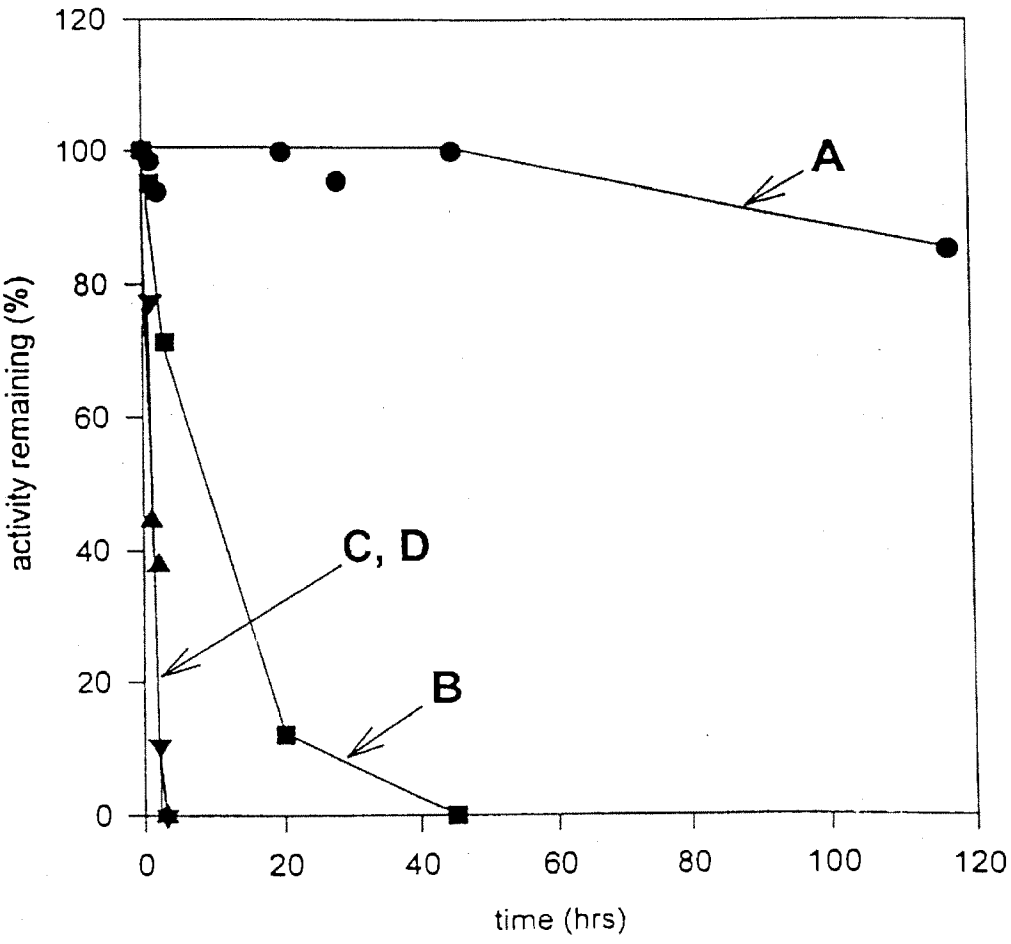

120
100
80
60
40
20
0
activity remaining (%)
0
20
40
60
80
100
120
time (hrs)
A
B
C, D

FIG. 16

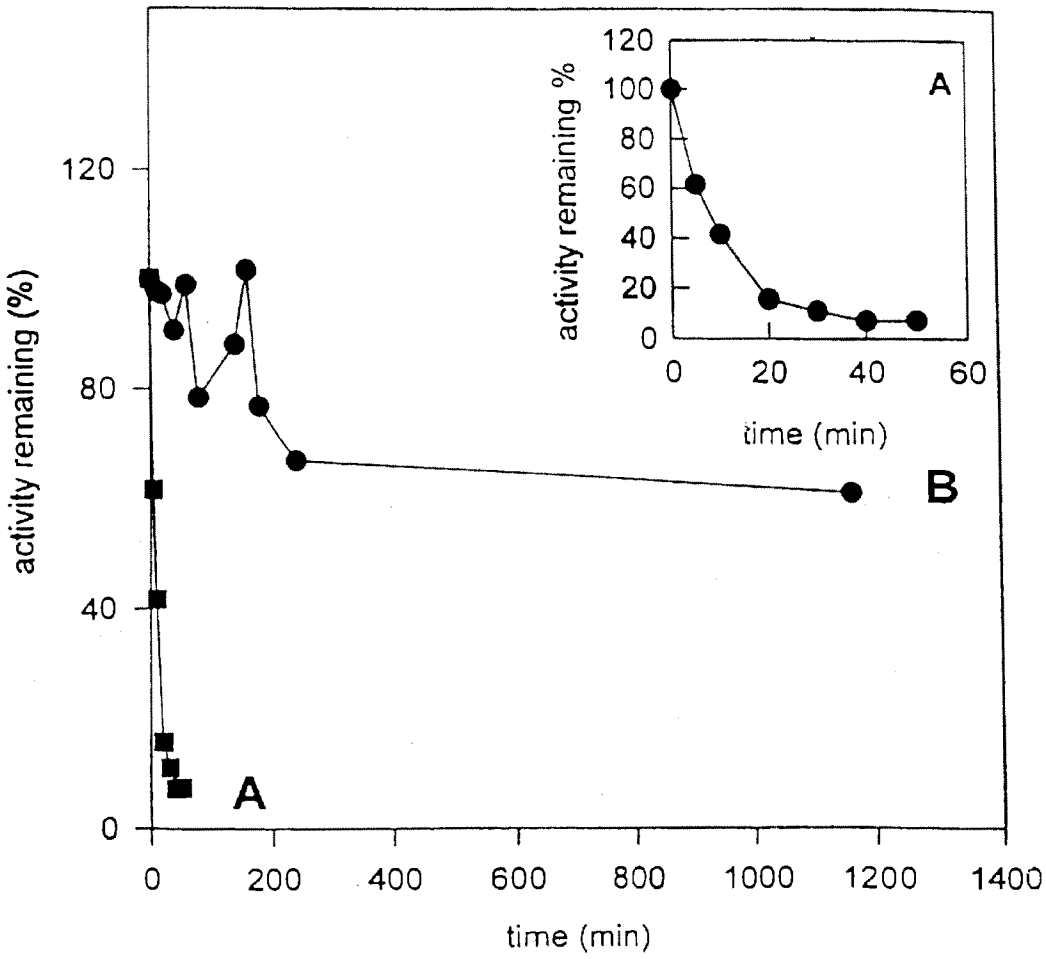

activity remaining (%)
120
80
40
0
0
200
400
600
800
1000
1200
1400
time (min)
A
B
activity remaining %
120
100
80
60
40
20
0
0
20
40
60
time (min)
A

FIG. 17

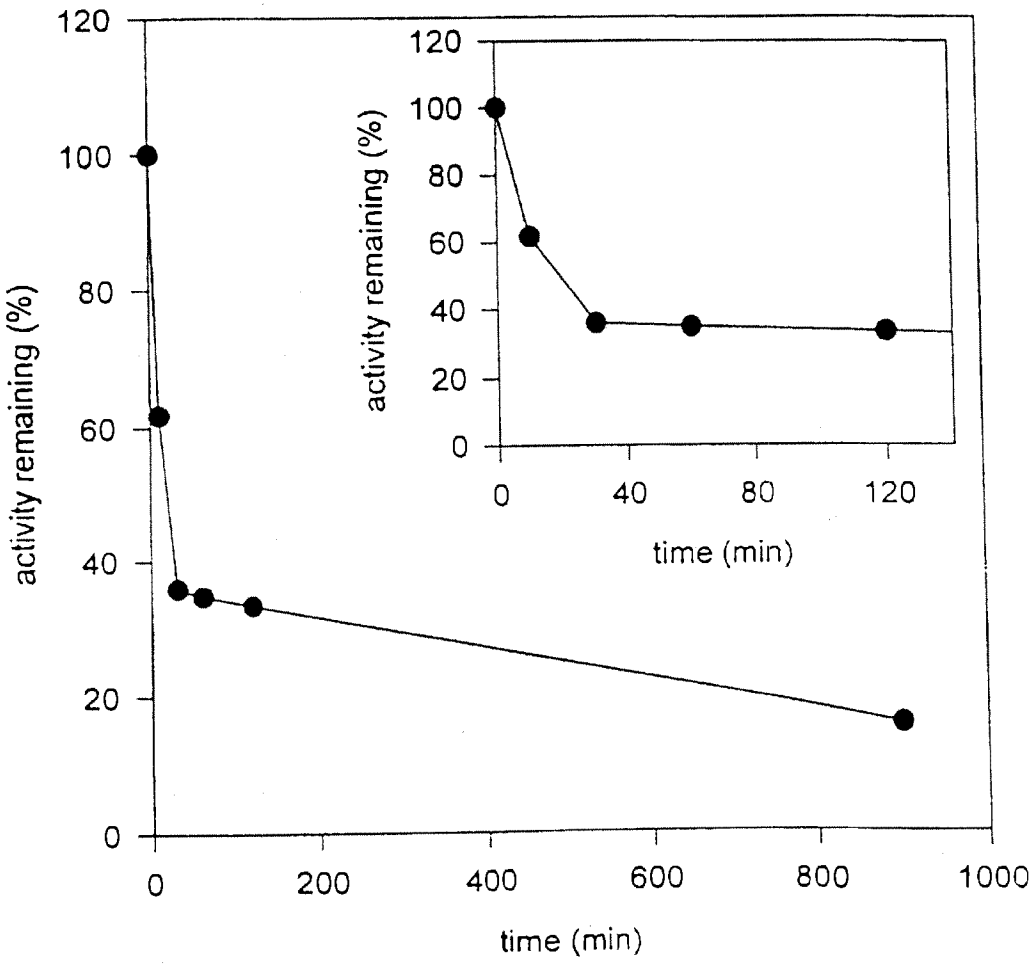

120
100
80
60
40
20
0
activity remaining (%)
0
200
400
600
800
1000
time (min)
120
100
80
60
40
20
0
activity remaining (%)
0
40
80
120
time (min)

FIG. 18A

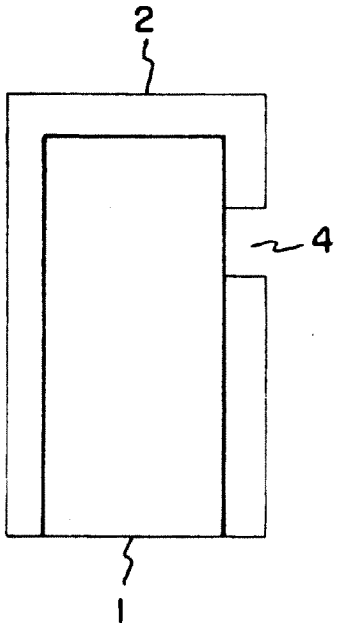


FIG. 18B

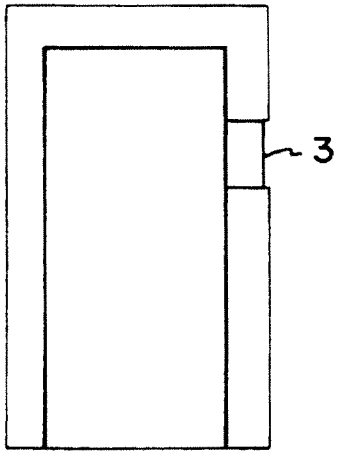


FIG. 19

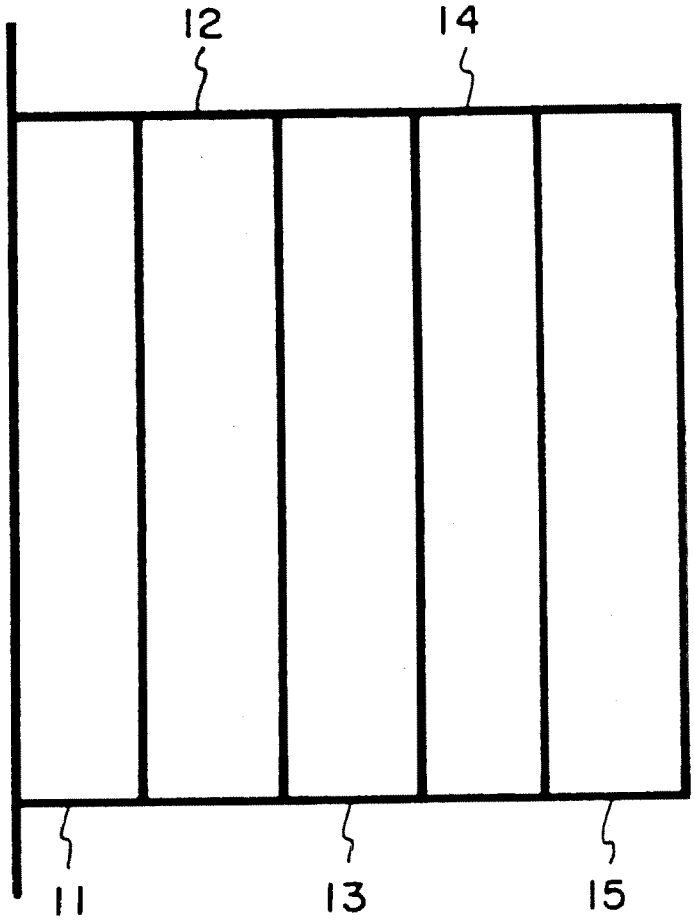


FIG. 21

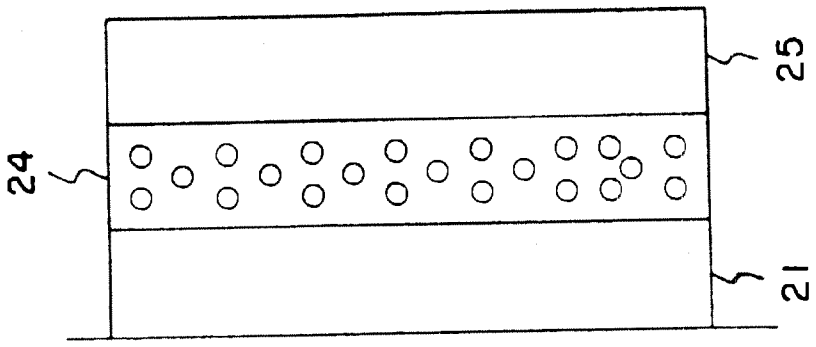
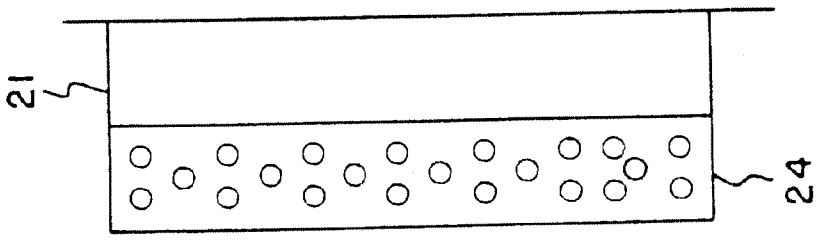


FIG. 20



ELECTROCHEMICAL ANALYTE SENSORS USING THERMOSTABLE SOYBEAN PEROXIDASE

This application is a continuation application under 37 CFR §1.53(b) of Ser. No. 09/291,230, filed on Apr. 13, 1999, now abandoned, which is a continuation of U.S. patent application Ser. No. 08/798,596 filed on Feb. 11, 1997, U.S. Pat. No. 5,972,199, which is a continuation-in-part of U.S. patent application Ser. No. 08/540,789 filed Oct. 11, 1995, U.S. Pat. No. 5,665,222, which application(s) are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to thermostable analyte sensors. More particularly, the invention relates to electrochemical sensors for the measurement of bioanalytes such as glucose and lactate. The inventive sensors include a thermostable peroxidase, such as soybean peroxidase, disposed on an electrode in a redox-compound-containing film. The sensor further includes a hydrogen peroxide-producing enzyme. The inventive sensors, for example in the measurement of blood glucose or lactate, operate for five days or more at 37° C., losing less than 10% of their sensitivity during continuous or intermittent operation.

BACKGROUND OF THE INVENTION

The assay of biochemicals, such as glucose and lactate, is important in medicine, biotechnology, and food processing (dairy and wine). Monitoring of glucose concentrations in fluids of the human body is of particular relevance to diabetes management. Monitoring of lactate in fluids of the human body is of relevance to diagnosis of trauma, of myocardial infarction, congestive heart failure, pulmonary edema, septicemia, hemorrhage, and others. Blood lactate levels above 7–8 mM are indicative of a fatal outcome. Bedside analyzers of lactate are useful in determining the response of patients to treatment, while in accidents and battle, they are useful in triage. Glucose assays are common in clinical practice and are applied in the diagnosis of Diabetes Mellitus and its management. Continuously or intermittently operating glucose sensors, including sensors implanted in the human body, are sought for the management of Type I diabetes, for example, for warning of imminent or actual hypoglycemia and its avoidance. Hypoglycemia can be fatal. For maintenance of diabetic patients at or near normal blood glucose levels, frequent or continuous monitoring of glucose is needed. Today, most Type I diabetic patients maintain their blood glucose at higher than normal levels, so as to reduce risk of fatal hypoglycemia. This is undesirable, as maintenance of higher than normal blood glucose levels has been shown to be a leading cause of blindness, kidney failure, neuropathy, and other complications of diabetes. It would, therefore, be useful to provide a glucose sensor that operates continuously or intermittently for a prolonged period of time to measure glucose in body fluid at 37° C. without substantial loss of sensitivity.

The present invention discloses material, structures, and methods enabling continuous operation of electrochemical sensors, for example, measuring glucose for more than one week or measuring lactate for more than 100 hours, with less than 10% loss in sensitivity.

SUMMARY OF THE INVENTION

Novel electrochemical sensors are presented, some of which are capable of operating at 37° C. continuously or intermittently, measuring biochemicals in body fluids with less than 10% loss of sensitivity in more than 100 hours of operation are described herein. The inventive sensors are relatively insensitive to electrooxidizable interferants, including ascorbate and acetaminophen.

The sensors of the invention include at least two enzymes, a thermostable peroxidase, such as soybean peroxidase, and a peroxidase-generating enzyme. In a preferred embodiment, redox centers of a thermostable peroxidase are electroreduced by electrons transported from a working electrode through a redox hydrogel in which the thermostable peroxidase is immobilized, preferably at a potential negative of 0.4 V versus the standard calomel electrode (SCE) and positive of −0.15 V (vs SCE). Most preferably, the thermostable peroxidase is coated on the electrode at a potential near 0.0V (vs SCE).

The preferred redox hydrogel comprises at least 20% by weight of water when in contact with a fluid to be assayed, and its redox centers are not leached by the assay fluid at 37° C. Non-leachable redox centers are bound to a polymer that forms the hydrogel upon water uptake. Preferably, the binding to the polymer is through covalent, electrostatic/ionic, or coordination bonds.

The redox centers of the peroxide-generating enzyme are preferably electrically insulated from the electrode, from the redox centers of the thermostable peroxidase, and from the redox centers of the redox hydrogel. The electrically insulated, peroxide-generating enzyme catalyzes reaction of a biochemical analyte, e.g., glucose or lactate, or a product of the analyte, with molecular oxygen. In the oxidation reaction, oxygen is reduced to hydrogen peroxide (H_2O_2).

The hydrogen peroxide-generating enzyme is preferably stabilized in a matrix. The preferred stabilizing matrices are macromolecular and inorganic. The most preferred matrices include silicon atoms, at least 50% of which are covalently linked to neighboring oxygen atoms, which are formed into a three-dimensional, crosslinked network. Such matrices can be made using a sol-gel polymerization process. The stabilizing matrix optionally includes a second polymer, which functions to further stabilize the insulated, peroxide-generating enzyme.

The peroxide-generating enzyme is preferably positioned behind or immobilized in a polymer that is at least tenfold, and preferably at least 100-fold, more permeable to oxygen than is the biochemical analyte to be measured. Examples of such polymers include silicone rubbers, produced by crosslinking a poly(dimethyl siloxane) derivative and cellulose acetate.

BRIEF DESCRIPTION OF THE FIGURES

Referring now to the drawings, wherein like reference numerals and letters indicate corresponding structure throughout the several views and where Examples 2–12 are for sensors described in Example 1:

FIG. **1** is a graph showing the dependence of current density on the weight fraction of the thermostable peroxidase in the sensing layer at 60 μg cm^{-2} loading (16 mM glucose, 1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. **2** is a graph showing the dependence of the electroreduction current density on the GOx loading (16 mM glucose, 1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. **3** is a graph showing the dependence of the calibration curves on the thickness of the cellulose acetate layer between the sensing layer and the immobilized glucose oxidase film. (A) 4 μl, (B) 8 μl, and (C) 28 μl of 0.25%

cellulose acetate (16 mM glucose, 1000 rpm, 37° C. pH7.3, in air, 0.00V (SCE));

FIG. **4** is a graph showing the potential dependence of the current density for a glucose sensor according to Example 4 (5 mM glucose, 1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. **5** is a graph showing the dependence of the current density on the dissolved oxygen concentration, according to Example 5 (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE)), (A) 5 mM glucose, (B) 5 mM lactate);

FIG. **6** is a graph showing the dependence of current density on the concentration of NaCl (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE)), (A) 5 mM glucose, (B) 5 mM lactate);

FIG. **7**A and FIG. **7**B are graphs showing the dependence of current density on pH,

FIG. **7**A shows data for 5 mM glucose;

FIG. **7**B shows data for 5 mM lactate (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. **8** is a graph showing the dependence of current density on the angular velocity (37° C., pH7.3, in air, 0.00V (SCE)), (A) SM glucose, (B) 5 mM lactate);

FIG. **9**A and FIG. **9**B are graphs showing the dependence of current density on temperature.

FIG. **9**A shows data for 5 mM glucose;

FIG. **9**B shows data for 5 mM lactate (1000 rpm, pH7.3, in air, 0.00V (SCE));

FIG. **10**A and FIG. **10**B are graphs showing the stability of thermostable peroxidase-based glucose and lactate sensors at different oxidase loadings: Figure (a) 1.3 μg; (b) 13 μg; (c) 26 μg; (d) 52 μg of immobilized glucose oxidase (FIG. **10**A); (a) 20 μg; (b) 40 μg; (c) 96 μg; (d) 160 μg of immobilized lactate oxidase (FIG. **10**B) (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. **11** is a graph showing glucose calibration curves before and after 280 hours of operation at 37° C. in the stability study of Example 8, (A) 26 μg GOx before stability experiment; (B) 26 μg GOx after stability experiment; (C) 1.3 μg GOx before stability experiment; (D) 1.3 μg GOx after stability experiment (1000 rpm, 37° C. pH7.3, in air, 0.00V (SCE));

FIG. **12** is a graph showing cyclic voltammograms of the glucose sensor before and after 280 hours of operation at 37° C.; (A) after stability experiment; (B) before stability experiment;

FIG. **13** is a graph of the time dependence of lactate oxidase activity in solution at 63° C.;

FIG. **14**A and FIG. **14**B are graphs showing time dependence of lactate oxidase-doped silica gel powder activity at 63° C.; FIGS. **14**A and **14**B(*a*): PVI-lactate oxidase-doped silica gel, FIG. **14**B(*b*): lactate oxidase-doped silica gel;

FIG. **15** is a graph showing time dependence of the activity of lactate oxidase in different environments at 50° C., (A) PVI-LOx-doped silica gel; (B) Lox-doped silica gel; (C) PVI-LOx in solution; (D) LOx in solution;

FIG. **16** is a graph showing time dependence of the activity of glucose oxidase in different environments at 63° C. (A), glucose oxidase in solution; (B) glucose oxidase-doped silica gel powder; the inset shows the short term behavior of (A);

FIG. **17** is a graph showing time dependence of PEI-lactate oxidase-doped silica gel powder activity at 63° C., inset shows the short term behavior;

FIG. **18**A is a diagrammatic representation of an electrode;

FIG. **18**B is a diagrammatic representation of an electrode with a sensing layer.

FIG. **19** is a diagrammatic representation of a sensing layer of a biosensor of the invention;

FIG. **20** is a diagrammatic representation of a two-layered sensing layer of a thermostable biosensor of the invention; and

FIG. **21** is a diagrammatic representation of a three-layered sensing layer of a thermostable biosensors of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Referring to the drawings in general and FIGS. **18**(*a*)–(*b*) in particular, the sensors of the invention typically include a non-corroding working electrode **1** which is substantially covered by an insulator **2** having a recess **4** to allow electrolytic contact between an analyte-containing fluid and the working electrode **1**. A film **3**, having a plurality of layers, is positioned within recess **4**. FIGS. **19**–**21** show three embodiments of film **3**. In general, film **3** includes a sensing layer **11**, **21** and a second enzyme layer **13**, **24**. Film **3** may also contain one or more optional layers, such as an electrically insulating layer **12** between the sensing layer **11** and second enzyme layer **13**; an analyte-transport controlling layer **14** to control the flow of analyte to the sensing layer **11**; and a biocompatible layer **15**, **25** for biocompatiblity of the sensor in body fluids.

Electrodes

The electrochemical sensors of the invention include at least two electrodes for contacting the electrolytic solution to be assayed. One of these electrodes is a working electrode **1**, which is made from non-corroding metal or carbon. A carbon working electrode may be vitreous or graphitic and can be made from a solid or a paste. A metallic working electrode may be made from platinum group metals, including palladium or gold, or a non-corroding metalically conducting oxide, such as ruthenium dioxide. The working electrode may be a wire or a thin conducting film applied to a substrate, for example, by coating or printing.

Typically, only a portion of the surface of the metallic or carbon conductor is in electrolytic contact with the analyte-containing solution. This portion is called the working surface of the electrode. The remaining surface of the electrode is isolated from the solution by an insulator **2**. Examples of useful insulators include polymers such as polyimides, polytetrafluoroethylene, polyhexafluoropropylene and silicones, also known as polysiloxanes.

In addition to the working electrode, the sensors of the invention also include a reference electrode or a combined reference and counter electrode (also termed a quasi-reference electrode or a counter/reference electrode). The reference or counter/reference electrode may be, for example, a silver/silver chloride electrode. If the sensor does not have a counter/reference electrode then it will include a separate counter electrode, which may be made from the same materials as the working electrode.

Sensors of the present invention have one or more working electrodes and one or more counter, reference, and/or counter/reference electrodes. One embodiment of the sensor of the present invention has two or more working electrodes. These working electrodes may be integrally connected or they may be kept separate.

Typically, for in vivo use the working electrodes of the present invention are implanted subcutaneously in the skin of a patient for direct contact with the body fluids of the

patient, such as blood. When multiple working electrodes are used, they may be implanted together or at different positions in the body. The counter, reference, and/or counter/reference electrodes may also be implanted either proximate to the working electrodes or at other positions within the body of the patient. Alternatively, the counter, reference, and/or counter/reference electrodes may be placed on the skin of the patient.

Sensing Layer

The portion of the working electrode that is not insulated is coated with a sensing layer **3**. The typical sensing layer **3** of the sensors of the present invention includes a redox polymer which is capable of swelling in water to form a redox hydrogel. Sensing layer **3** also typically includes an enzyme which is capable of catalyzing a reaction of the analyte or an analyte-generated compound. Preferably, that enzyme is immobilized in the redox polymer. In one embodiment of the invention, the sensor contains a peroxidase which is preferably a thermostable peroxidase such as soybean peroxidase. The peroxidase-containing hydrogel forms a sensing layer that transduces a flux of H_2O_2 into an electrical signal.

The redox polymer contains redox centers that are bound to the polymer by covalent, coordinative, or electrostatic bonds; the latter resulting preferably from interactions of charged sites of the polymer with an oppositely charged redox species. Preferably, the redox mediator is covalently or coordinatively bound to the redox polymer, although it may also be immobilized by entrapment or by electrostatic bonding.

The redox centers are preferably non-leachable. For redox species electostatically bound to the polymer, the absence of leaching generally requires that the charged redox species has, at the pH of the analyte solution, a charge of at least two, and preferably three or more. For example, if the redox species is a cation, it preferably has at least a +2 charge, and is preferably +3 or better. The greater the charge of the redox species, the slower the leaching. Similarly, if the redox species is an anion and the binding to the polymer is electrostatic, then it should have a −2 charge and is preferably −3 or better. Preferably, most of the redox centers (at least about 90%) remain electrode-bound for at least 14 days at 37° C.

The redox polymer is designed to swell in water and dilute aqueous solutions. The water uptake should add at least 10 weight percent, and preferably 20 weight percent, to the weight of the dry polymer. The thermostable peroxidase in the redox polymer layer is not substantially leached by water or by a physiological buffer solution at 37° C. The redox polymer layer containing the thermostable peroxidase can be conveniently made, for example, as described by Gregg and Heller, *J. Phys. Chem.* 95:5970 (1991), in U.S. Pat. No. 5,262,035, and in copending U.S. patent application Ser. No. 08/540,789, filed Oct. 11, 1995.

In general, the redox polymer contains redox centers that are stable and have a redox potential between about −0.1 V (vs SCE) and about +0.6V (vs SCE). The preferred range is between about −0.1V (vs SCE) and about +0.4V (vs SCE). Poising the electrode at a potential where O_2 is not electroreduced is desirable; otherwise the measured signal resulting from the electroreduction of H_2O_2 to water must be corrected for the background caused by electroreduction of dissolved O_2. Furthermore, it is also desirable to poise the electrode at a potential where the rate of electrooxidation of other interferants, such as urate, ascorbate, and acetaminophen, is not significant, so that correction for their electrooxidation will not be required.

Examples of useful redox centers that can be bound to the polymer of the sensing layer are described in U.S. Pat. Nos. 5,264,104; 5,356,786; 5,262,035; and 5,320,725, herein incorporated by reference. Typically, the redox mediators are metal complexes, particularly of osmium, ruthenium, iron and cobalt, or organic redox compounds, such as quinones and other compounds having a quinoid structure. The preferred redox centers exchange electrons rapidly with each other. One example of the preferred redox centers are osmium transition metal complexes with one or more ligands having a nitrogen-containing heterocycle such as 2,2'-bipyridine, 1,10-phenanthroline or derivatives thereof. In particular, it has been determined that osmium cations complexed with two ligands containing 2,2'-bipyridine, 1,10-phenanthroline, or derivatives thereof, the two ligands not necessarily being the same, form particularly useful redox centers in the sensors of the present invention. Preferred derivatives of 2,2'-bipyridine for complex formation with the osmium cation are 4,4'-dimethyl-2,2'-bipyridine and mono-, di-, and polyalkoxy-2,2'-bipyridines such as 4,4'-dimethoxy-2,2'-bipyridine, where the carbon to oxygen ratio of the alkoxy groups is sufficient to retain solubility of the transition metal complex in water.

One useful group of redox polymers is derived from poly(4-vinyl pyridine) and is made by complexing between one third and one fifteenth of the pyridine rings with $[Os(bpy)_2Cl]^{+/2+}$ where bpy is 2,2'-bipyridine, or with $[Os(phen)_2Cl]^{+/2+}$ where phen is 1,10-phenanthroline, and where, preferably, some of the pyridine rings of the polymer are quaternized by reaction with 2-bromoethylamine. Useful polymers are also derived from poly-(N-vinyl imidazole) (termed PVI), poly(acrylamide co-4-vinylpyridine), and poly(acrylamide co-4-vinylimidazole).

To produce the inventive sensors, a hydrogen peroxide sensing layer, containing a thermostable peroxidase, is prepared by the dropwise mixing of solutions of the peroxidase, the redox polymer, and a crosslinker on a clean electrode surface. These solutions are allowed to dry and cure. The solutions may also be premixed, and a droplet of the premixed solution may be placed on the electrode. An example of a useful crosslinker is poly(ethylene glycol) diglycidyl ether with a molecular weight between 300 and 600, preferably 400 and 600. Another useful crosslinker is the bis N-hydroxysuccinimide ester of poly(ethylene glycol)-α,ω-dicarboxylic acid.

Second Enzyme Layer

A second enzyme layer **13** on the working electrode catalyzes the reaction of O_2 with a substrate whereby H_2O_2 is produced. The substrate is generally the analyte to be assayed by the sensor. Alternatively, the substrate is a reaction product of the analyte to be assayed, for example the product of hydrolysis of the analyte. The second enzyme used in layer **13** is also termed herein the peroxide-generating enzyme. In the inventive sensors, the redox centers of the peroxide-generating enzyme are prevented from being reduced or oxidized directly by electron exchange with the redox polymer of the sensing layer. Prevention of such oxidation or reduction is accomplished by methods discussed hereinbelow.

First, prevention of oxidation/reduction of the peroxide-generating (second) enzyme by the redox polymer can be intrinsic to the second enzyme. For example, the second enzyme may include a sufficiently thick, natural, electrically insulating protein or glycoprotein layer over its reaction center or centers. Such a layer prevents electron transfer to or from the redox polymer. In this case, no further means for preventing reduction or oxidation of the second enzyme by the redox polymer is required.

Inorganic Polymeric Matrix Immobilization and Stabilization

Alternatively, the H_2O_2-generating (second) enzyme is immobilized in a non-conducting inorganic or organic polymeric matrix to prevent electron exchange between its redox centers and the redox polymer. The immobilizing matrix is preferably highly permeable to O_2 and usually is more permeable to O_2 than to the analyte or to its precursor. The preferred immobilizing matrices are those in which the second enzyme is stabilized, becoming itself thermostable through immobilization, as discussed more fully below.

The sol-gel polymerization process provides a method for the preparation of an inorganic polymeric matrix (e.g., glass) by the polymerization of suitable monomers at or near room-temperature. Suitable monomers include alkoxides and esters of metal and semiconducting elements, with preferred metals and semiconductors comprising Si, Al, Ti, and Pb. The most preferred monomers include silicon and have a silicon to oxygen ratio of between about 1:2 and about 1:4.

Enzymes can be immobilized in silica and other inorganic polymeric matrices made by sol-gel processes, involving, for example, the hydrolysis of tetramethoxysilane or another polyalkoxysilane that may contain one or more silicon atoms. Condensation of the resulting silanol in the presence of the enzyme results in entrapment of the enzyme. This process has been referred to as sol-gel immobilization.

Enzymes can be immobilized in inorganic polymeric matrices to prevent electrical contact between the immobilized second enzyme and the redox polymer and/or electrode. Furthermore, binding of enzymes in silica or other inorganic polymeric matrices formed from sol-gels can stabilize the enzyme. Entrapment of glucose oxidase, a glycoprotein, in silica sol-gel greatly improves the stability of the enzyme, which retains activity when heated in water to 98° C. for 10 minutes.

When lactate oxidase, which is not a glycoprotein, was similarly immobilized by the sol-gel process in silica, the enzyme was not particularly thermostable. However, when lactate oxidase is dissolved in an aqueous buffer solution in which poly(N-vinyl imidazole) is co-dissolved, and the lactate oxidase-poly(N-vinyl imidazole) mixture is immobilized in the silica by the sol-gel process, a uniquely stable, immobilized lactate oxidase is obtained. The stabilized lactate oxidase can be heated in water to 90° C for 10 minutes and still retain enzymatic activity.

Poly(N-vinyl imidazole), a polycation at pH 7, binds at this pH to enzymes such as lactate oxidase, that are polyanions at pH 7. Thus, the addition of a particular polymer to a particular enzyme can greatly increase the stability the enzyme. In the case of lactate oxidase, addition of polyethyleneimine, also a polybasic polymer and also multiply protonated at pH 7, in place of poly(N-vinyl imidazole) improved stability of the enzyme, although not as much as the addition of the preferred polymer, poly(N-vinyl imidazole).

If the enzyme is a polycation or polyanion at a particular pH, a polymeric polyanion or polycation, respectively, can be coimmobilized in an inorganic polymeric matrix formed from a sol-gel to stabilize the enzyme. The stabilized enzyme can then be used at higher temperatures and/or for longer durations than would be possible if the enzyme were immobilized alone in the sol-gel.

After gelling, the silicas, in which the peroxide-generating, now thermostable, second enzyme is immobilized, are hard materials that can be ground into fine powders. These powders are then dispersed in highly O_2-permeable polymers, further discussed hereinbelow, to form a peroxide-generating second layer **13** of the inventive sensors. The peroxide-generating layer **13** preferably has the following desired properties: thermostability; absence of electron transfer between the redox polymer of the sensing layer and the redox centers of the enzyme of the second enzyme containing H_2O_2-generating layer, i.e. absence of oxidation or reduction of the redox polymer by redox centers of the second enzyme; and high O_2 permeability.

Alternatively, when electron transport between the redox polymer of the sensing layer and the peroxide-generating second enzyme is avoided because of intrinsic electrical insulation of the H_2O_2 generating enzyme or by immobilization of the enzyme in an electrically insulating matrix (such as the inorganic polymeric matrices described above), it is practical to incorporate the second enzyme in the peroxidase-containing sensing layer. In this case, the sensing layer includes the redox polymer, electrically connecting the thermostable peroxidase (first enzyme) to the electrode, and also includes the peroxide-generating enzyme (second enzyme), the redox centers of which are not electrically connected to the redox polymer or to the electrode.

Alternatively, the peroxide-generating enzyme layer **24** can be formed directly over the sensing layer **21** (see FIGS. **20** and **21**), but only when the peroxide-generating enzyme is intrinsically insulated or immobilized in an electrically insulating matrix. If the H_2O_2 generating enzyme is neither intrinsically insulating nor immobilized in an insulating matrix, an H_2O_2-permeable, electrically insulating layer **12** can be placed between the sensing layer **11** and the peroxide-generating (second) enzyme layer **13** to achieve the desired insulation.

The peroxide-generating (second) enzyme layer is formed by any of the following methods. Particles, such as silica-containing particles in which the second enzyme is stabilized, can be dispersed in a polymer, such as a silicone rubber solution or silicone rubber precursor solution. A droplet of this mixture is then applied to the working electrode. Alternatively, the peroxide-generating layer is formed by adding a cross-linking agent to the second enzyme and applying a droplet containing the second enzyme and its crosslinker to the working electrode. Useful cross-linking agents include, for example, poly(ethylene glycol) diglycidyl ether (PEGDGE). For cross-linking of glycoproteins such as glucose oxidase, the enzyme is oxidized with $NaIO_4$, forming reactive aldehyde functions condensing with surface amines, such as those of enzyme lysines. The preferred method for producing a peroxide-generating layer containing glucose oxidase, when the enzyme is not immobilized in a silica sol-gel, is the latter, $NaIO_4$ method. For peroxide-generating layers containing lactate oxidase, which is also not immobilized in a silica sol-gel, cross-linking of its mixture with poly(N-vinyl imidazole) with PEGDGE is preferred. The second enzyme may also be immobilized with glutaraldehyde or glutaraldehyde and a protein such as albumin.

An enzyme stabilized by the silica sol gel matrix can be ground to a find powder and dispersed in a silicone, preferably in an elastomeric silicone, and most preferably in a water-based elastomeric silicone precursor. This dispersion is then applied to the thermostable peroxidase layer to form a hydrogen peroxide-generating layer. Silicone is a preferred binder in this layer due to its oxygen permeability.

Electrically Insulating Layer

Another means for preventing oxidation/reduction of the peroxide-generating (second) enzyme is the addition of an electrically insulating membrane **12** that is permeable to

H_2O_2 between sensing layer **11** and second enzyme layer **13**. The insulating membrane can be inorganic or organic, but preferably is an organic polymer such as cellulose acetate. The second enzyme may be immobilized in a layer **13** over the polymeric membrane.

Preferably, the insulating membrane **12** is permeable to the small H_2O_2 molecule but is not permeable to the larger, readily oxidizable, potentially interfering ascorbate, acetaminophen or urate species. Preferably, the ratio of the permeability of layer **12** to H_2O_2 and one or more of ascorbate, acetaminophen, and urate is greater than 2:1. Useful, selectively H_2O_2 permeating polymer films include dense cellulose acetate, cured Nafion™, and nylon. Using known, standard methods, dense, small pore size films of cellulose acetate or nylon are cast from cyclohexanone or tetrahydrofuran; Nafion™ films are made from alcohol and alcohol-water emulsions and curing.

Analyte-Transport Controlling Layer

If the second enzyme is not immobilized, or when it is desired to broaden the dynamic rauge of the sensor so that the sensor will respond, preferably linearly, to higher concentrations of the analyte, then an analyte-transport controlling layer **14** is placed between the solution that is being analyzed and second enzyme layer **13** of the sensor. This analyte-transport controlling layer **13** functions to contain the second enzyme and thereby avoid its loss, and/or to limit the flux of the analyte or second enzyme substrate to the second enzyme layer.

It is of essence that this second membrane be permeable to O_2, the co-reactant in the enzyme-catalyzed generation of H_2O_2 in the presence of the substrate. It is also of essence that the ratio of permeabilities of O_2 and of the substrate or its precursor through this second membrane be large enough for oxidation or dehydrogenation of the substrate in the reaction catalyzed by the second enzyme with the concurrent formation of water, a reaction whereby O_2 is consumed. Because the concentration of O_3 in saturated aqueous solutions is well below the usual concentrations of analytes such as glucose or lactate in biological fluids, including fluids in the body of animals, analyte-transport controlling layers **14** that are much more permeable to O_2 than to the substrate or its precursor are preferred.

Generally, membranes or polymers for which the permeability coefficient of molecular oxygen is greater than 10^{-8} cm^3 (STP) cm $cm^{-2}s^{-1}$ (cm Hg)$^{-1}$ at 25° C. are preferred. (Explanation of the permeability units is given in *Polymer Handbook, Second Edition,* J. Pandrup and E. H. Immergut, Editors, John Wiley & Sons, New York, 1975, pages III-229–231.) Polymers for use in the analyte-transport controlling layer **14** are those that are highly permeable to O_2, preferably those in which the ratio of O_2 and substrate (or substrate precursor) permeabilities is greater than 10:1, and more preferably greater than 100:1. It is well known that the permeability of O_2 in silicones, for example in silicone rubbers, formed by cross-linking poly(dimethyl siloxanes), is particularly high. Thus, silicones are useful polymers for this layer. Another useful material is cellulose acetate.

Biocompatible Layer

For in vivo use, the sensor is preferably coated with a biocompatible film **15** that is permeable to the substrate converted in the reaction catalyzed by the second enzyme. This biocompatible film is placed on the solution side of the sensor.

The preferred biocompatible layer **15** is formed of a hydrogel, e.g., a polymeric composition which contains more than 20% by weight of water when in equilibrium with a physiological environment such as living tissue or blood. An example is a cross-linked derrivative of polyethylene oxide, e.g., a tetraacrylated derrivative of polyethylene oxide. The polymeric compositions are non-toxic, non-immunogenic, non-thrombogenic, and otherwise compatible with living tissue of animals.

A biocompatible layer **15** is not necessary if the analyte-transport controlling layer **14** or the second enzyme layer **13** are biocompatible. For example, many solid silicones are biocompatible and therefore require no additional biocompatible layer (see FIG. **20**, in contrast the embodiment of the invention shown in FIG. **21** includes a biocompatible layer **25**).

Stability of the Sensor

An important performance criterion of the inventive sensor is the rate of loss of sensitivity. This rate depends on the concentration of the analyte, because loss in sensitivity is usually first noticed at high analyte concentrations. At higher analyte concentrations, where complete conversion of the incoming analyte flux to H_2O_2 requires the presence of more of the active second enzyme, the system is very sensitive to inadequate substrate conversion in the second enzyme layer. The rate of loss of sensitivity can be reduced, even when the second enzyme is not particularly thermostable, by limiting the flux of analyte to the second enzyme layer **13** by using an analyte-transport controlling layer **14** and limiting thereby the analyte concentration.

Loss of sensitivity can also be limited and even avoided for a period of days or weeks, by incorporating in the second enzyme layer a large amount of enzyme that even if part of it becomes inactive, all of the analyte flux is still transduced by the remaining second enzyme into an H_2O_2 flux. If a thermostable second enzyme, such as an enzyme entrapped in an inorganic polymeric matrix, is used to form the second enzyme layer **13**, sensors of unprecedentedly good stability and long life can be built and can be further improved either by incorporating a large excess of the thermostable second enzyme, much more than needed to convert the analyte flux into an H_2O_2 flux, or by limiting the analyte flux, or by using both techniques cooperatively.

The preferred sensors are also stable for long periods of time at temperatures of 37° C. or greater. Preferably, the sensor is stable for 100 hours or more at this temperature with a drop in the sensor's output of 10% or less. Preferably, the sensor and the enzymes within the sensor are stable at 30° C., more preferably at 40° C., and most preferably at 50° C. or 60° C. or higher.

Other Sensors

Many of the principles of the invention can be used in sensors other than the peroxidase sensors described hereinabove. In particular, the immobilization of a second enzyme in an inorganic polymeric matrix formed using the sol-gel process can be used effectively in the enzyme sensor described in U.S. Pat. No. 5,593,852 herein incorporated by reference. In the sensor, a layered structure similar to that depicted in FIGS. **19–21** is used. However, in the sensing layer an analyte-sensitive enzyme is used rather than a peroxidase. For example, glucose oxidase or glucose dehydrogenase is present in the sensing layer if the analyte is glucose or lactate oxidase is used if the analyte is lactate.

These sensors often have a second enzyme layer which is functions as an interferent-eliminating layer. This layer contains one or more enzymes which catalyze reactions of interferents, such as ascorbate, urate, acetaminophen. Enzymes for use in this layer include peroxidases and lactate oxidase (when glucose is the analyte) or glucose oxidase (when lactate is the analyte). These enzymes can be immobilized in an inorganic polymeric matrix as described here-

inabove and used to form a second enzyme layer. Furthermore, these enzymes may be stabilized by immobilization in the inorganic polymeric matrix either alone or in conjunction with a polyelectrolytic polymer as described hereinabove.

EXAMPLES

The invention will be further defined by reference to the following Examples. These examples are not intended to limit the scope of the invention, but rather to illustrate some of the invention's specific embodiments.

Example 1

Preparation of 4-layered Sensors

Soybean peroxidase (SBP) was obtained from Harlan Bioproducts for Science, Indianapolis, Ind., High grade, 130 pyrogallol units/mg (Cat. No: SP04 Lot No: SPLO515). The lactate oxidase (LOx) was obtained from Genzyme, Boston, Mass., Lot # D50293, Cat. # 70-1381-01, EC 1.1.2.3 from *Acerococcus viridans,* 37.0 units/mg of powder. Poly (ethylene glycol) diglycidyl ether (PEGDGE) was obtained from Polysciences, Inc., Warrington, Pa. A 30% hydrogen peroxide solution was obtained from Aldrich, and diluted solutions were prepared daily.

Electrodes

Vitreous carbon electrodes, 3 mm in diameter, were polished and cleaned using 3 grades of alumina slurry: 5, 1, and 0.3 microns, with sonication and rinsing between grades. Each polished and cleaned electrode was tested in PBS by scanning the potentials of interest (−0.4 V to +0.4V vs. SCE) to ensure that the electrochemistry was featureless. The four-layered sensors were prepared as follows:

Hydrogen Peroxide Sensing Layer

A redox polymer, PVP-bpy-Os (or POs-EA.), was synthesized by partially complexing the pyridine nitrogens of poly-(4-vinylpyridine) with $Os(bpy)_2Cl^{+/+2}$, and then partially quaternizing the resulting polymer with 2-bromoethylamine, by the method described in Gregg and Heller, 1991, *J Phys. Chem.* 95:5970. The osmium-containing redox centers allow for electrical communication between the carbon surface and the peroxidase heme centers, and the pyridinium-N-ethylamine functions enhance hydration and provide primary amines for cross-linking. The ratio of unmodified pyridines to osmium-complexed pyridines to ethylamine quarternized pyridines was 3.3:1.0:0.8.

The first layer, the hydrogen peroxide sensing layer, was prepared by placing droplets of 2 μl PVP-bpy-Os (5 mg/ml), 2 μl soybean peroxidase (5 mg/ml), and 1 μl of the crosslinker PEGDGE (2.5 mg/ml) on the carbon surface. The droplets were mixed on the surface of the electrode with the tip of a syringe. The electrode was then allowed to dry for 16 hours at room temperature. The resulting dark purple film appeared to be well spread and uniform. The dried electrode was washed in PBS for 20 minutes at 1000 rpm, rinsed with water, and permitted to dry at room temperature.

Hydrogen Peroxide Transport-Limiting Layer

To prepare the second layer, 4 μl of 0.5% cellulose acetate (Sigma, approximately 40% acetate content) in cyclohexanone (Aldrich) was placed atop the dried and washed hydrogen peroxide sensing layer. The electrode was permitted to dry for two hours at room temperature. The resulting film appeared to be well spread and uniform.

Second Enzyme Layer (Without Sol-gel Silica)

To immobilize the glucose oxidase (GOx) enzyme, two different immobilization procedures were used. For GOx, a solution of 100 μl of 20 mg/ml of GOx in water was prepared. Glucose oxidase (GOx) was obtained from Sigma, ED 1.1.3.4 from *Aspergillus niger* type X-S, 198 units/mg solid, 75% protein. After the addition of 50 μl of 12 mg/ml $NaIO_4$ in water, the enzyme-periodate solution was incubated at room temperature and in the dark for two hours, following the procedure described in Vreeke et.al., 1995, *Anal Chem.* 67:4247. A volume of 4 μl of the incubated mixture was placed atop the cellulose acetate film to form an immobilized GOx analyte sensing layer, which was permitted to dry overnight.

For LOx, a mixture of 10 μl of 20 mg/ml LOx, 20 μl of 10 mg/ml PVI, and 10 μl of 5 mg/ml PEGDGE in water was prepared. A volume of 4 μl of the LOx mixture was placed atop the cellulose acetate film to form an immobilized LOx analyte sensing layer, which was permitted to dry overnight.

Analyte Transport-Limiting Layer

The fourth polymeric layer, an analyte transport-limiting layer of cellulose acetate, was prepared and applied to the analyte sensing layer as described above for the hydrogen peroxide transport-limiting layer. A volume of 4 μl of 0.5% cellulose acetate in cyclohexanone was placed atop the immobilized oxidase layer (analyte sensing layer), and permitted to dry for two hours.

The four-layered electrode was then washed in PBS for 25 minutes and rinsed with water.

Example 2

Varying Amounts of Soybean Peroxidase

The 4-layered electrodes were prepared as described for Example 1, but varying the enzyme weight fraction of soybean peroxidase in the crosslinked redox polymer of the hydrogen peroxide sensing layer or by varying the total loading of SBP. Current measurements were collected using a Princeton Applied Research model 273 potentiostat/galvanostat in a 3-electrode cell. All measurements were performed using a 20 mM phosphate buffer (pH 7.3) containing 0.1 M NaCl, except measurements in which pH dependence was studied. In experiments where pH was varied, 2M solutions of HCl or NaOH were added to the phosphate buffer solution.

Glucose and lactate solutions were prepared by diluting a stock 2M solution in phosphate buffer. All experiments were run at 37° C. under air in 100 ml of phosphate buffer, unless otherwise indicated. The cell contained a rotating glassy carbon working electrode, a saturated calomel reference electrode (SCE), and a platinum counter electrode, isolated from the bulk solution by a Vycor™ frit. The stability measurements were run in a stirred cell, the rotating disk experiments were performed using a Pine RDE4 potentiostat, with an MSRX speed controller, an X-Y-Y' plotter, and a VWR 1165 refrigerated constant temperature circulator. The rotating electrode experiments were performed at 1000 rpm, unless otherwise noted.

Results:

As shown in FIG. **1**, for a given mass of redox polymer (POsEA), the current density peaked between 40 wt % and 60 wt % of SBP. For a 50 wt % electrode, the current density reached a plateau at a loading of 60 $\mu g/cm^2$. Although current densities were independent of loading above 60 $\mu g/cm^2$, the response times increased with increased loading. For this reason, the preferred sensing layer contained 50 wt % SBP at a total loading of 60 $\mu g/cm^2$.

FIG. **2** shows the dependence of current densities on the amount of glucose oxidase immobilized on the electrode. The current densities at 4 mM glucose reached a plateau at GOx loadings of 375 $\mu g/cm^2$. Electrodes made with LOx

exhibited similar behavior with a current density plateau at a loading of 600 $\mu g/cm^2$.

Example 3

Varying Thickness of First Transport-controlling Layer and Fourth Analyte-transport-limiting Layer

The hydrogen peroxide transport-controlling layer functions to control the transport of hydrogen peroxide from the analyte sensing layer to the hydrogen peroxide sensing layer. This layer also serves to electrically isolate the POsEA of the hydrogen peroxide sensing layer from the immobilized oxidase layer, that is, to prevent "short-circuiting" of the sensor through "wiring" of the oxidase.

When the immobilized oxidase of the analyte sensing layer is not sufficiently insulated from the hydrogen peroxide sensing layer, redox centers of the oxidase and osmium centers of the POs-EA are electrically connected. This results in the "short-circuiting" of the electrodes. As described in Vreeke and Rocca, 1996, *Electroanalysis* 8:55, a symptom of "short-circuiting", which is often seen only at high substrate concentrations, is the suppression of the catalytic reduction current upon increasing substrate concentration. In the present experiments, "short circuiting" was diagnosed by poising the electrode at +0.5 V (SCE), where the hydrogen peroxide was not catalytically electroreduced on the "wired" peroxidase layer, and injecting 10 mM substrate. Flow of an oxidation current indicated that the electrode had a short circuit.

In the electrodes of Example 1 formed with application of 4 μl of 0.25% cellulose acetate, "short circuiting" by "wiring" of the oxidase by POsEA was effectively prevented, in contrast to electrodes prepared with thinner cellulose acetate films. (data not shown). Electrodes made without the inner cellulose acetate layer invariably exhibited short-circuiting, and the effect was often quite substantial. For example, a typical glucose electrode made without this insulating layer showed current suppression at concentrations higher than 1 mM glucose, and the current was suppressed to less than 50% of its maximum value at a concentration of 20 mM. This observation demonstrates the necessity of the insulating layer in this type of sensor.

Membranes, having a range of thickness, were obtained by using solutions of different cellulose acetate concentrations, and applying various volumes of these. The thinnest membranes were made by applying 1 μl of 0.25 weight % cellulose acetate. The thickest membranes were made by applying 4 μl of 0.5 weight % cellulose acetate solution to the surface.

While the presence of the inner cellulose acetate layer effectively prevented short-circuiting, the sensitivity of the electrodes dropped by about 50%. The sensitivity loss is attributed to less efficient collection of the H_2O_2 generated by the oxidase-catalyzed reaction.

An analyte transport-limiting layer forms a fourth polymeric layer. As described for Example 1, this layer was made of cellulose acetate, and provides substrate transport control, thereby defining the dynamic range of the sensor. Increasing the thickness of the outer cellulose acetate layer decreased the sensor's sensitivity, but increased the apparent K_m. FIG. **3** shows the response of an electrode over-coated with varying amounts of cellulose acetate: (A) 4 μl; (B) 8 μl; (C) 28 μl of 0.25% cellulose acetate. Increasing the thickness of the outer cellulose acetate layer from 4 μl (A) of 0.25% cellulose acetate to 8 μl (B) decreased the sensitivity by 50%, but increased the apparent K_m from 1 mM to 2 mM glucose. The response time ($t_{10/90}$) for electrodes with an outer cellulose acetate layer of 8 μl of 0.25% was less than 2 minutes.

Example 4

Potential Dependence of the Electrodes

The redox polymer of the electrodes of Example 1, POs-EA, has a formal redox potential of +278 mV (vs. SCE). (Gregg and Heller, 1991, *J Phys. Chem.* 95:5970) FIG. **4** shows the dependence of current density on the applied potential for a glucose electrode of Example 1. Between 0.20 V and +0.35 V (vs. SCE), the current density was independent of applied potential. At applied potentials positive of +0.40 V, a reduction current was no longer observed. At applied potentials more negative than –0.20 V, oxygen reduction effects are observed.

Example 5

Oxygen Dependence of the Electrodes

In nature, the reduced form of an oxidase requires oxygen for reoxidation and production of hydrogen peroxide. FIG. **5** shows the dependence of the current density on the partial pressure of the dissolved oxygen for the electrodes of Example 1. At 37 C, the concentration of oxygen in an air-saturated aqueous solution is about 0.2 mM (See, Hodgman Ind.: *Handbook of Chemistry and Physics,* 44th Ed, The Chemical Rubber Publishing Co., Cleveland, Ohio, 1963, page 1706).

For glucose electrodes, even at very low oxygen concentrations, the current densities were lower only by about 20% than those in oxygen saturated solutions. For oxygen concentrations of 0.04 mM or higher, i.e., in solutions saturated with atmospheric pressure argon containing only 4 volume % oxygen, the current density was independent of the oxygen concentration.

For lactate electrodes, however, at low oxygen partial pressures, the dependence of current density on oxygen concentration was more substantial. This difference is explained by the different immobilization methods for GOx and LOx. The sodium periodate crosslinked GOx layer has lower oxygen permeability than that of the PEGDGE crosslinked PVI-LOx layer. At high (5 mM) lactate concentrations, the current density became independent of the partial pressure of oxygen only when the volume % of oxygen reached its volume % in air (21%). For lower lactate concentrations, it is expected that the current will be less dependent on oxygen partial pressure as a lower oxygen flux suffices for the quantitative conversion of lactate to hydrogen peroxide and pyruvate.

Example 6

Salt and pH Dependence of the Electrodes

FIG. **6** shows the dependence of current density on the concentration of NaCl for both GOx (A) and LOx (B) electrodes of Example 1. Although the current densities were somewhat higher at low salt concentrations, the electrodes were virtually insensitive to salt concentrations up to 1M NaCl. Increasing the salt concentration from 0.1M to 1M decreased the current density by less than 10%.

FIGS. **7**A and **7**B show the dependence of the current density on pH for both GOx (FIG. 7A) and LOx (FIG. 7B) electrodes of Example 1. For the glucose electrodes, the current density was near its maximum between pH 4.5 and

pH 6.5, the plateau matching the pH dependence of GOx activity in solution, which exhibits maximal activity near pH 5.5. For lactate electrodes, the current density reached its maximum at pH 4.0, differing from the pH dependence of LOx activity in solution, which exhibits maximal activity at pH 6.5. Both glucose and lactate electrodes were irreversibly deactivated below pH 3.0.

Example 7

Rotation Rate and Temperature Dependence

FIG. **8** shows the dependence of current density on the angular velocity of the rotating electrodes for both GOx (A) and LOx (B). The current density was practically independent of rotation rate. For example, in the case of the glucose electrode, increasing the rotation rate from 50 rpm to 1000 rpm increased the current density by less than 5%.

FIGS. **9**A and **9**B show the dependence of the current density on temperature for both electrodes, FIG. **9**A shows data for 5 mM glucose; and FIG. **9**B shows data for 5 mM lactate.

Example 8

Long Term Operational Stability

The stability of the current density of the sensors of Example 1 during continuous operation at 37° C. was a strong function of oxidase loading. As shown in FIG. **10**A, the higher the GOx loading, the better the operational stability at 37° C. An electrode loaded with 1.3 μg of GOx (a) had an operational half life of 40 hours, while an electrode loaded with 52 μg of GOx (d) maintained 100% of its initial current density for over 100 hours and lost only 10% of its initial current density after 200 hours, and less than 25% after 280 hours. FIG. **11** shows the current response before and after the long term operational stability experiments for glucose electrodes at both high and low loadings. While the electrode with a 1.3 μg GOx loading lost more than 90% of its current density at all glucose concentrations after **100** hours of operation at 37° C., the electrode with 26 μg GOx loading demonstrated no loss of current density at low concentrations, and a loss of only 25% of current density at glucose concentrations greater than 5 mM after 200 hours of operation. Cyclic voltammetry revealed that the electrodes did not lose electroactive osmium throughout the long term stability experiments (FIG. **12**), and operational stability was maintained as long as the amount of active enzyme sufficed to oxidize all of the glucose flux.

Similarly, FIG. **10**(B) shows that the higher the LOx loading, the better the operational stability at 37 C. While the electrode with a 20 μg LOx loading lost more than 80% of its current density at all lactate concentrations after 120 hours of continuous operation at 37 C, the electrode with 160 μg LOx loading demonstrated no loss of current density at low concentrations, and a loss of only 10% of current density at lactate concentrations greater than 5 mM after 160 hours of operation. Cyclic voltammetry revealed that the electrodes did not lose electroactive osmium throughout the long term stability experiments, and operational stability was maintained as long as the amount of active enzyme sufficed to oxidize all of the lactate flux (data not shown).

Example 9

Selectivity Against Interferents

Interferents such as ascorbate, urate, and acetaminophen are oxidized at POsEA coated carbon electrodes. These interferents decrease the catalytic hydrogen peroxide electroreduction current. Nevertheless, no current change was observed with the electrodes of Example 1, upon the addition of urate up to 0.5 mM or acetaminophen up to 1.0 mM, both in the presence and absence of substrate. Ascorbate was electrooxidized only at a negligibly slow rate. For example, at 1 mM glucose, an injection of 0.1 mM ascorbate caused a decrease of less than 1% in the current density, and, at 64 mM glucose, injection of 0.1 mM ascorbate caused a decrease of less than 0.1% in the current density.

Example 10

Preparation and Activity Test for Lactate Oxidase-doped Silica Gel Powder

Preparation of PVI-LOx-doped Silica Gel Powder

0.15 g of tetramethylorthosilicate (TMOS, Cat. # 34,143–6, Aldrich, Milwaukee, Wis.) was added to a small vial. The vial was placed into an ice-bath, and stirred at approximately 600 rpm. 36 mL of 2.44 mM HCl was added and the solution was stirred for 10 min. A vacuum was then applied to the vial for an additional 10 minutes to eliminate the methanol produced in the sol process. The vacuum was released, and pH of the solution was adjusted to pH 5.1 by adding 20 mL of 20 mM phosphate buffer (pH 7.4).

In separate small vial, 6 mg of lactate oxidase (LOx, from aerococcous viridens, 41 units/mg, Cat. #1381, Genzyme, Cambridge, Mass.). 180 mL of 10 mM 4-(2-hydroxyethel)-1-piperazineethane-sulfonic acid (HEPES) buffer solution (pH 7.5), and 20 mL of 20 mg/mL of aqueous poly(1-vinylimidazole) were mixed as described by Timothy J. Ohara, Ravi Rajagopalan and Adam Heller, *Anal. Chem.* 1994, 66, 2451–57. This solution was added to the TMOS-containing solution and was stirred for 1 minute. A vacuum was applied to the stirred mixture until a gel formed. The vacuum was released and the gel was rinsed with 2 mL of distilled water three times. The gel was then soaked in 2 mL of distilled water overnight at 4° C. The water was removed from the vial and the gel was allowed to dry at room temperature overnight. The dried gel was collected and ground to a powder with a mortar and pestle.

Qualitative Activity Test for PVI-LOx-doped Silica Gel Powder

For a qualitative activity test of lactate oxidase-doped silica gel at room temperature, peroxide test strips (Reflectoquant® 16974 Peroxide Test, Merck, Darmstadt, Germany) were used. 1–2 mg of lactate oxidase-doped silica gel powder was applied to the active area of the test strip. 20 μL of 1 M lactate (prepared in phosphate buffer solution, pH 7.4) was applied to the same area of the test strip. The presence of lactate oxidase activity was indicated by the appearance of a blue color on the active area of the strip.

Quantitative Activity Test for PVI-LOx-doped Silica Gel Powder

In the presence of oxygen, a lactate oxidase catalyzes the oxidation of lactate to pyruvate. In the process, hydrogen peroxide is formed stoichiometrically. The enzymatically formed hydrogen peroxide can be quantitatively measured using a peroxidase linked assay system, which causes the formation of a quinoneimine dye. This dye can be measured spectrophotometrically at 564 nm.

Preparation of Solutions for PVI-LOx-doped Silica Gel Powder Activity Test

Reaction mixture I was prepared by combining the following solutions:

(a) 6.0 mL 0.2 M 3,3-dimethylglutarate [$HO_2CCH_2CH_2C(CH_3)_2CO_2H$]—NaOH buffer, pH 6.5;

(b) 3.0 mL peroxidase (from horseradish, Sigma Chemical Inc, St. Louis, Mo.), 50 units/mL in water;

(c) 3.0 mL 15 mM 4-aminoantipyrine in water;

(d) 3.0 mL 0.5 M substrate; and

(e) 9.0 mL distilled water.

A stock solution of 0.5% N,N'-dimethylaniline was prepared by mixing 0.2 g into 100 mL of water. A stock solution of the enzyme diluent was prepared by adding 66 mg of FAD into 4 mL of a 10 mnM KH_2PO_4—NaOH buffer solution, pH 7.0. Lactate oxidase solutions were prepared at a concentration of 1 mg/mL in enzyme diluent. A stock solution of enzymatic reaction stopper was prepared by dissolving 0.25 g of dodecylbenzenesulfonic acid sodium salt [$C_{12}H_{25}C_6H_4SO_3Na$] in 100 mL of distilled water. All chemicals were purchased from Aldrich Chemical Company (Milwaukee, Wis.) unless otherwise mentioned.

Activity Test Procedure for PVI-LOx-doped Silica Gel Powder

1.0 mg of lactate oxidase-doped silica gel powder was weighed into a 3 mL cuvette. 100 μL of distilled water was used to wash the powder down from the inside walls to the bottom of the cuvette. The cuvette was then capped with parafilm and placed in a temperature bath set at 63° C. for the experimental period of time. After this period of time, the cuvette was removed from the bath, and 100 μL of enzyme diluent was added. The cuvette was then placed into a 37° C. temperature bath for another five minutes.

A solution with a 4:1 ratio of reaction mixture I to 0.5% dimethylaniline was prepared and allowed to equilibrate at 37° C. One milliliter of this solution was added to the cuvette. This mixture was agitated and allowed to react for exactly 10 minutes at 37° C. Immediately after this incubation, 2.0 mL of the reaction stopper were added and the absorbance at 564 nm (As) was measured.

The procedure was repeated, using 1.0 mg of enzyme-free silica gel powder in place of the lactate oxidase-doped silica gel powder. The same procedure was followed thereafter and the blank absorbance (Ab) was measured. Therefore, the net absorbance of the lactate oxidase-doped silica gel powder was determined by ΔA (ΔA=As−Ab). If ΔA was greater than 0.6, the assay was repeated, and the weight of the lactate oxidase-doped silica gel powder was adjusted accordingly.

Results for PVI-LOx-doped Silica Gel Powder

FIG. **13** shows the activity decrease of lactate oxidase in solution over time. The enzyme had a half-life of 6.8 minutes at 63° C., which was in agreement with the previously published value (H. Minagawa, N. Nakayama, and S. Nakamoto, 1995, *Biotechnology Letters* 17(9):975).

FIGS. **14**A and **14**B show the comparison of the enzyme activity loss of PVI-lactate oxidase-doped silica gel powder with that of lactate oxidase-doped silica gel powder at 63° C. FIG. **14**A shows PVI-lactate oxidase-doped silica gel. FIG. **14**B compares PVI-lactate oxidase-doped silica gel (a) with lactate oxidase-doped silica gel (b). At 63° C., the LOx-doped silica gel powder (b) had the same half-life as that of LOx in solution. However, the PVI-LOx-doped silica gel powder FIG. **14**B(*a*) and FIG. **14**A maintained 60% of its initial activity after 900 minutes at 63° C.

FIG. **15** shows the time dependence of the activity loss for lactate oxidase in different environments at 50° C. At this temperature, the PVI-LOx-doped silica gel powder (A) maintained 85% of its initial activity after 120 hours, whereas the LOx-doped silica gel powder (C) had a half-life of only 10 hours. Both the LOx in solution (D) and the PVI-LOx in solution (C) demonstrated a half-life of less than one hour at this temperature.

The PVI-LOx-doped silica gel powder maintained 100% of its initial activity after 11 days of soaking in water at room temperature.

Example 11

Preparation and Activity Test for Glucose Oxidase-doped Silica Gel Powder

Preparation of GOx-doped Silica Gel

1.527 g of tetramethylorthosilicate(TMOS) was added to a small vial. The vial was placed into an ice-bath, and the solution was stirred at approximately 600 rpm. 360 mL of 2.44 mM HCl was added and the solution was stirred for 10 min. A vacuum was applied to the vial for an additional 10 minutes to eliminate the methanol produced in the sol process. The vacuum was released and the pH of the solution was adjusted to pH 5.1 by adding 100 mL of 20 mM phosphate buffer (pH 7.4). In a separate small vial, 67 mg of glucose oxidase (GOx from *Aspergillus niger,* Cat. # G-7141, 183 units/mg, from Sigma, St. Louis, Mo.) and 540 mL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (pH 7.5) were mixed. This solution was added to the TMOS-containing solution and was stirred for 1 minute. A vacuum was applied to the stirred mixture until a gel formed. The vacuum was released, and the gel was allowed to dry overnight at room temperature. The dried gel was first collected and then ground to a powder with a mortar and pestle.

Qualitative Activity Test for GOx-doped Silica Gel

For a qualitative activity test of glucose oxidase-doped silica gel at room temperature, peroxide test strips (Reflectoquant® 16974 Peroxide Test, Merck, Darmstadt, Germany) were used. 1–2 mg of glucose oxidase-doped silica gel powder was applied to the active area of the test strip. 20 μL of 1 M glucose (prepared in phosphate buffer solution, pH 7.4) was applied to the same area of the test strip. The presence of glucose oxidase activity was indicated by the appearance of a blue color on the active area of the strip.

In order to test the activity of the glucose oxidase-doped silica gel powder at higher temperatures, the powder was immobilized onto a glass slide according to the following procedure. 26 mg of Bermocoll® EHM-100 (Berol Nobel AB, Stenungsund, Sweden), 1 mL of cyclohexanone and 1 mL of water were mixed in a small vial. The vial was heated and agitated over a flame for 5 minutes. The solution was allowed to settle and the white micelle fraction was collected. 90 μL of the micelles and 10 mg of the glucose oxidase-doped silica gel powder were mixed and then stirred for 5 minutes. 10 μL of the mixture was applied to a glass slide and allowed to dry overnight. The glass slide was immersed in a temperature bath for 5 minutes at the experimental temperature. The slide was removed and allowed to dry. 10 μL of 2 M glucose was applied to the immobilized micelles. The active area of the peroxide testing strip was placed on top of the immobilized micelle area. The appearance of a blue color on the strip indicated the presence of glucose oxidase activity.

Quantitative Activity Test for GOx-doped Silica Gel

The quantitative activity test of glucose oxidase was performed exactly as that for lactate oxidase except a 1 M glucose solution was used in place of the 1 M lactate solution and glucose oxidase-doped silica gel powder was used in place of lactate oxidase-doped silica gel powder.

Qualitative Results for GOx-doped Silica Gel

The glucose oxidase-doped silica gel powder retained activity after immersion in water for 5 minutes at 40, 50, 60, 70, 84, and 92° C., while similarly immobilized glucose oxidase in DAEA Dextran, Heparin, Lactitol, Gafquats, and Dextran Sulfate showed no activity retention at temperatures higher than 70° C.

The glucose oxidase-doped silica gel powder was then tested for activity at 92° C. for longer time periods. Activity

was retained for up to 20 minutes, after which, the micelles washed off of the slides. At 75° C., activity was retained for over 60 minutes.

Quantitative Results for GOx-doped Silica Gel

FIG. **16** shows the comparison of the enzyme activity loss of glucose oxidase-doped silica gel powder (B) with that of glucose oxidase in solution (A) at 63° C. At this temperature, the GOx-doped silica gel powder (B) maintained 60% of its initial activity after 1200 minutes, while the half-life of GOx in solution (A) was 6.5 minutes, as shown in the figure inset.

Example 12

Preparation and Activity Test for PEI-LOx-doped Silica Gel

Polyethyleneimine (PEI) is chemically similar to PVI and has been shown to stabilize enzymes. PEI-LOx-doped silica gel powder was prepared according to the same procedure as that for PVI-LOx-doped silica gel powder. FIG. **17** shows the activity loss over time of PEI-LOx-doped silica gel powder at 63° C. After 900 minutes, the PEI-LOx-doped silica gel powder maintained 18% of its initial activity.

Example 13

Preparation and Activity Test of PVI-LOx Adsorbed onto Colloidal Silica

PVI-LOx was also adsorbed onto colloidal silica according to the following procedure. 1.69 mg of LOx was dissolved in 23 μL of 20 mg/mL of PVI. This solution was mixed with 33 μL of colloidal silica (34 wt. % suspension in water, Aldrich, Milwaukee, Wis.). The resulting solution was allowed to dry. The solid was first collected and then ground to a powder in a mortar and pestle. The PVI-LOx adsorbed silica had the same half-life as LOx in solution at 63° C.

Example 14

Layered Sensor with Sol-Gel Silica Immobilized GOX Particles in Silicone

A 0.29 mm polyimide insulated gold wire (polyimide insulation thickness 0.02 mm, gold wire OD 0.25 mm) was etched galvanostatically in a KCN solution as described by E. Csöregi et.al., *Anaytical. Chemistry* 66:3131–3138, 1994, to form about 100 μm deep recess. The following sequence of droplets were deposited in the recess to form a four-layered structure. The innermost layer contained a hydrogen peroxide-sensing soybean peroxidase and redox polymer. The second layer was a hydrogen peroxide permeable insulating layer. The third layer was a silicone layer in which sol gel silica-immobilized glucose oxidase containing particles were dispersed. The fourth, cellulose acetate layer was oxygen permeable and glucose flux reducing.

To form these four layers on the electrode, the following procedure was used. The redox polymer solution prepared as described for Example 1 was used at a concentration of 10 mg/mL. Soybean peroxidase (SBP) in deionized water was used at a concentration of 10 mg/mL. PEGDGE was used at 5 mg/mL. The redox polymer, SBP, and PEGDGE solutions were mixed at a 2:2:1 ratio. Ten droplets of about 5 nL each of the mixed solution were sequentially deposited within the recess to form the sensing layer, which was cured at 55° C. for 20 minutes.

The solution from which the second layer, the hydrogen peroxide permeable membrane, was cast, consisted of 0.5% weight % cellulose acetate (40% acetylated) in cyclohexanone. One 5 nL droplet of this solution was applied to the cured sensing layer in the recess.

The hydrogen peroxide generating enzyme layer (third layer) was made of 0.5 parts of the sol-gel immobilized glucose oxidase of Example 15, 1 part of Dow Corning 3-5025 silicone water based elastomer that was thoroughly mixed and dispersed in 9 parts of water by grinding in an agate mortar for 30 minutes. Three drops of this mixture were applied on the cellulose acetate layer, and cured at 50° C. for ten minutes.

The fourth layer was made of 2 weight % cellulose acetate (40% acetylated) also containing 1.6 weight % TWEEN 80 in cyclohexane, of which one drop was applied onto the third layer.

When the electrode, produced as described above, was poised at −0.045V (SCE) in a phosphate buffer solution at pH 7.0 (20 mM phosphate, 0.14 M NaCl), the current in the absence of glucose was 0.1 nA. When the glucose concentration was brought to 5 mM, the current increased in less than 20 seconds to 3.6 nA. Further increases of the concentration to 10 mM resulted in respective currents of 6.6 nA and 8.5 nA.

Example 15

Two Layered Glucose Electrode

Recessed palladium electrodes were prepared as described by E. Csöregi et.al., *Anaytical. Chemistry* 67:1240, 1995, for gold electrodes, except that the solution in which the palladium was etched under galvanostatic control was not a potassium cyanide solution, but 6M Hcl. The recess formed was about 100 μm deep. Aqueous solutions of 10 mg/mL soybeanperoxidase, 10 mg/mL POs-EA and 5 mg/mL PEGDGE were prepared.

In a small vial, 20 μL of the peroxidase solution, 20 μL of the POs-EA solution, and 10 μL of the PEGDGE solution were mixed. 5 nL of this mixed solution were applied to the palladium surface. The solution was allowed to dry, and the application and drying steps were repeated seven times. The electrode was allowed to cure overnight at room temperature.

In a small vial, 0.1 g of Dow Corning Water Based Elastomer Silicone (#3-5025, Lot #LL059026) and 0.9 g of water were thoroughly mixed. 0.95 g of this mixture and 0.05 g of glucose oxidase-doped silica gel powder were mixed. 5 nL of this mixture were applied on top of the "wired" soybean peroxidase layer of the recessed palladium electrode. The electrode was then cured for ten minutes at 50° C.

The electrode was poised at −0.045 V (SCE) in a phosphate buffer solution at pH 7.0 (20 mM phosphate, 0.14 M NaCl) and its current was measured as a function of the glucose concentration in the solution. The apparent K_m of the electrode was 5 mM glucose, i.e., half of the maximum current was reached at 5 mM glucose. The response times, i.e., the rise of the current from 10 to 90% of the maximum current, were less than one minute. The sensitivity of the electrode was 0.8 nA/mM glucose.

We claim:

1. An electrochemical hydrogen peroxide sensor comprising:

an electrode; and

a thermostable peroxidase immobilized on and electrically connected to the electrode;

the sensor being capable of operation at 37°C. with less than 2% loss of sensitivity per hour of continuous operation.

2. The hydrogen peroxide sensor of claim **1**, wherein the thermostable peroxidase is soybean peroxidase.

3. The hydrogen peroxide sensor of claim **1**, further comprising a redox polymer disposed on the electrode and coupled to the thermostable peroxidase.

4. The hydrogen peroxide sensor of claim **3**, wherein the thermostable peroxidase is crosslinked to the redox polymer.

5. The hydrogen peroxide sensor of claim **3**, wherein the redox polymer comprises an osmium cation.

6. The hydrogen peroxide sensor of claim **5**, wherein the osmium cations are coordinatively bound to the redox polymer.

7. The thermostable peroxidase of claim **1**, wherein the peroxidase is non-leachable.

8. The hydrogen peroxide sensor of claim **1**, further comprising a biocompatible layer disposed over the thermostable peroxidase and the electrode.

9. The hydrogen peroxide sensor of claim **1**, further comprising a second enzyme capable of catalyzing the production of hydrogen peroxide in the presence of an analyte.

10. The hydrogen peroxide sensor of claim **9**, wherein the second enzyme is disposed on the electrode but electrically insulated from the electrode.

11. The hydrogen peroxide sensor of claim **9**, wherein the second enzyme is insulated from the electrode by the intrinsic protein structure of the second enzyme.

12. The hydrogen peroxide sensor of claim **9**, wherein the second enzyme is insulated from the electrode by a hydrogen peroxide-permeable membrane or polymer.

13. The hydrogen peroxide sensor of claim **1**, wherein the sensor is capable of operation at 50° C. with less than 5% loss of sensitivity per hour of continuous operation.

14. The hydrogen peroxide sensor of claim **13**, wherein the sensor is capable of operation at 50° C. with less than 2% loss of sensitivity per hour of continuous operation.

15. The hydrogen peroxide sensor of claim **1**, wherein the sensor is adapted for in vivo use.

16. The hydrogen peroxide sensor of claim **1**, wherein the sensor is capable of operation at 37° C. for five days with less than 10% loss of sensitivity.

17. The hydrogen peroxide sensor of claim **1**, wherein the sensor is capable of operation at 37° C. with less than 2% loss of sensitivity per hour of continuous operation with intermittent measurements of analyte concentration.

18. The hydrogen peroxide sensor of claim **1**, wherein the sensor is adapted for subcutaneous implantation.

19. An electrochemical hydrogen peroxide sensor comprising:

an electrode; and

a thermostable peroxidase immobilized on and electrically coupled to the electrode;

wherein, in the presence of hydrogen peroxide, the thermostable peroxidase generates an electric signal.

20. An electrochemical sensor comprising:

an electrode;

a thermostable peroxidase disposed on the electrode; and

a redox polymer disposed on the electrode configured and arranged to couple the peroxidase to the electrode.

* * * * *

EXHIBIT 2

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Glucose ENFET doped with MnO_2 powder

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Abstract

In this study, a glucose-sensitive enzyme field-effect transistor (ENFET) coimmobilized with glucose oxidase (GOD) and manganese dioxide (MnO_2) have been investigated. The biomembrane of the ENFET was immobilized on the amorphous tin oxide/indium tin oxide glass structure extended sensitive gate, which used as a disposable transducer of a glucose biosensor. MnO_2 was used as a catalyst which can catalyze the hydrogen peroxide and produced H_2O and O_2. Coimmobilizaion of glucose oxidase and manganese dioxide was found to be useful for extending the dynamic measured range of glucose concentration to 360 mg/dl (eq. 20 mM). The result shows that the dynamic range of the output signal is strongly dependent on pH value of measuring environments, and the measurement in the alkali buffer solutions shows a higher response and wider dynamic range. Additionally, the different immobilized layers of MnO_2 have been studied. The MnO_2, which be immobilized in outer cross-linking layer of bovine serum albumin, shows better results than immobilized in GOD layer or gultaraldehyde covalent layer.

Keywords: Extended gate field effect transistor (EGFET); Enzyme field effect transistor (ENFET); Amorphous tin oxide; Glucose oxidase (GOD); Manganese dioxide (MnO_2)

1. Introduction

Since the first reported enzyme biosensor (ENFET) based on ion-sensitive field effect transistors (ISFETs) [1], substantial research efforts were undertaken to improve the performance characteristics of the ENFETs developed. Until now, there are almost two dozen papers dealing with glucose ENFETs which suffer from many problems [2].

Normally glucose oxidase hydrolyzes glucose according to the following reactions:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\beta\text{-D-glucose oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$\text{D-glucono-}\delta\text{-lactone} \rightarrow \text{D-gluconate} + H^+ \quad (2)$$

ISFET sensors measure the glucose concentration by detecting the pH variation due to the hydrogen ions that are generated by the dissociation of gluconic acid. However, because of the low dissociation constant ($pK_a \cong 3.8$) [3], ISFET glucose sensors show low sensitivities. Generally, the sensitivities at the physiological pH value are limited to only some millivolts per decade [4]. Hence the ISFET drift, which is an inherent characteristic of ISFETs, becomes a important topic. The glucose concentration in human blood is normally about 5 mM, reaching 20 mM and more for diabetics. However, the concentration of oxygen, does not exceed 0.5 mM. Because of the unfavourable concentration ratio of glucose and oxygen in real blood, the dynamic range of the biosensor is usually limited by oxygen and dose not exceed several mM. Since, the oxygen in the sensor membrane is consumed by the enzyme reaction, the oxygen concentration is needed high enough for a better linearity between output voltage and the glucose concentration. Moreover, the hydrogen peroxide, one of the by-products of the glucose oxidation, acts an inhibitor of glucose oxidase which causes the lower sensitivity and bad repeatability in the steady measurement system of glucose ENFET.

Sudoh et al. employed pre-electrolysis method to enrich the oxygen of the glucose solution, which the oxygen is generated by electrolysis of the solution before monitoring

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[5]. The output signal was linearly proportional to the glucose concentration up to 500 mg/dl by the supplement of oxygen generated by electrolysis of the solution. Seo et al. and Lee et al. employed a Pt electrode actuator on the ISFET sensitive gate to electrolyze the hydrogen peroxide [2,6]. The sensor with the Pt electrode actuator exhibits a wide dynamic range that from 1 to 10 mM. Saito et al. used an external BSA membrane, which is highly cross-linked by glutaraldehyde, to restrict glucose diffusion to expand the measuring range and make a stable response in a low buffer capacity solution [7]. The sensor outputs shows a good linear relationship with up to 300 mg/dl glucose concentration. Shul'ga et al. added the potassium ferricyanide, which used as an oxidizing substrate in the biocatalytic oxidation of glucose, into the phosphate and TRIS buffer to perform the glucose ENFET measurement [8]. Depending on the concentration of ferricyanide the glucose ENFET shows a 10–100 times increase of the biosensor response and a substantial extension of its dynamic range.

In this paper a glucose sensor based on H^+-ion-sensitive field effect transistor (ISFET) has been realized in combination with a MnO_2-doped glucose oxidase membrane. Zheng and Guo brought forward the following procedures and reactions of H_2O_2 catalyzed by MnO_2 [9]:

$$MnO_2 + H_2O \rightarrow MnO(OH)_2 \quad (3)$$

$$MnO(OH)_2 \rightarrow MnO^{2+} + 2OH^- \quad (4)$$

$$MnO^{2+} + H_2O_2 + 2OH^- \rightarrow MnO_4^{2-} + H_2O \quad (5)$$

$$MnO_4^{2-} \rightarrow MnO_2 + O_x \quad (6)$$

Where MnO_2 was used as a catalyst which can catalyze the hydrogen peroxide and produce H_2O and O_2. In addition to the reduction of H_2O_2 concentration in the biolayer, the product, oxygen, can be recycled for glucose oxidation reaction. The MnO_2 doping position and the pH value of working have been investigated.

2. Experimental

2.1. Chemicals and materials

The β-D-glucose oxidase (GOD) EC 1.1.3.4 from *Aspergillus niger*, bovine serum albumin from Serva and γ-aminopropyl triethoxysilane (3-APTS, 99%) were purchased from Sigma. Glutaraldehyde (GA, 25% aqueous solution) was purchased from Acros Organics. Manganese dioxide powder (99.9%) was obtained from Tekstart (Hsinchu, Taiwan). All other regents were in reagent grade and were used without further purification. Distilled water was used for all the electrolytes and the buffer solutions. Tin oxide thin films were formed by the RF sputtering system (tin oxide target, 99.9%) at a substrate temperature of 150°C. The ITO glasses (50–100 Ω/sq; ITO coating thickness, 230 Å) were supplied by the Wintek Corporation.

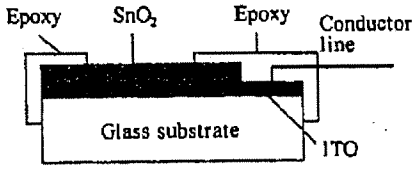

Fig. 1. Cross-section of SnO_2/ITO glass sensing structure.

2.2. Sensor Fabrication

2.2.1. Solid-state part

In this study, the glucose ENFET is based on a separative extended gate ISFET (EGFET) structure. The sensitive part of the separative EGFET is shown as Fig. 1. The SnO_2 thin film was deposited by using sputtering method with a thickness of 2000 Å. Before the glass was deposited SnO_2, it was washed in methyl alcohol and DI water for 20 and 10 min, respectively. The SnO_2/ITO glass EGFET shows a linear pH response about 57 mV/pH between pH 2.4 and pH 11.2 [10].

2.2.2. Enzyme immobilization

The procedure for preparation of separative structure of ENFET is as follows.

1. Cleaning: The separative structure of EGFET was cleaned by distilled water.
2. Silanization: There is no amino group on our sensitive film, so we use 3-APTS to modify tin oxide (SnO_2) substrate. The procedure is represented as follow [11,12]:

$$SnO_2 \xrightarrow{\text{silanizaton}} SnO_x\text{–}Si(CH_2)_3NH_2$$

3. Activation by glutaraldehyde: Glutaraldehyde (1%) is also used extensively to immobilize enzyme molecules onto a carrier substance bearing amino group. The procedure is represented as follow:

$$SnO_x\text{–}Si(CH_2)_3NH_2 \xrightarrow{\text{glutaraldehyde}} SnO_x\text{–}Si(CH_2)_3N{=}CH\text{–}(CH_2)_3\text{–}CHO$$

4. Coupling of the enzyme and cross-linking: The GOD (40 mg) was dissolved in 1 ml of a 0.1 M K–P buffer solution (pH 7.0). A 1.5 μl part of the solution was cast onto the gate region and then addition of 1 μl of the glutaraldehyde was followed to chemically cross-link the membrane. The procedure is represented as follow:

$$SnO_x\text{–}Si(CH_2)_3N{=}CH\text{–}(CH_2)_3\text{–}CHO \xrightarrow{\text{enzyme}} SnO_x\text{–}Si(CH_2)_3N{=}CH\text{–}(CH_2)_3\text{–}CH{=}N\text{–}(\text{enzyme})$$

5. The outer BSA membrane doping with MnO_2: An amount of 10 mg MnO_2 was dissolved in 300 mg/dl BSA and 6% glutaraldehyde (1:1) solution. A 1 μl part of the solution was cast onto the enzyme membrane.

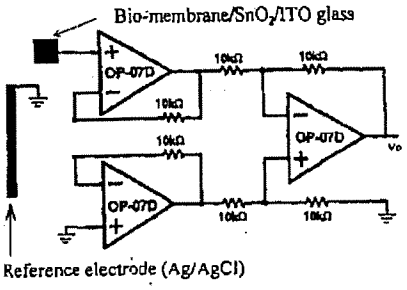

Fig. 2. Separative extended ENFET measurement circuit with instrumentation amplifier LT1167.

In the study of the effect of MnO_2 doping position, the outer BSA membrane was not immobilized, and the MnO_2 was doped in activation or GOD with 10 mg/ml MnO_2.

2.3. Measurement

A readout circuit based on an instrumentation amplifier LT1167 is shown in Fig. 2. The measurement configuration consists of a separative extended gate with biomembrane and the Ag/AgCl reference electrode. The instrumentation amplifier, LT1167, was a transducer and the small output voltage will depend on the pH value. HP3478A and HP VEE program were designed and used as an Y–T recorder to record the voltage variation with time. All measuring temperature of our experiments are in 25°C, 5 mM phosphate–KOH buffer.

3. Results and discussion

3.1. Glucose ENFET response

In this study, a separative sensitive gate of biomembrane/SnO_2/ITO glass structure was used as a disposable biochemical transducer. This structure has advantages of light insensitivity, easier fabrication processes than traditional ISFET and lower cost than SOS structure ISFETs or silicon based EGFET [10]. Fig. 3 shows the pH response of separative sensitive structure with biomembrane of sensitivity 58.3 mV/pH between pH 2 and 10.

Figs. 4 and 5 show typical time response curve for the glucose ENFET without and with the outer BSA membrane. The glucose ENFET was immersed in blank buffer solution for 1min and then immersed in glucose solution. As the ENFET are measured in blank buffer, it shows a drift that not exceeds to 1 mV for 1 min. The glucose ENFETs without and with BSA membrane show response time of 5 and 12 min, respectively. The glucose ENFET, which has outer MnO_2-doped BSA membrane, shows a good linearity up to 360 mg/dl, because the O_2 concentration in biomembrane was greatly rose by MnO_2 catalyzing H_2O_2. The relationship between the sensor output and glucose concentration is shown in Fig. 6.

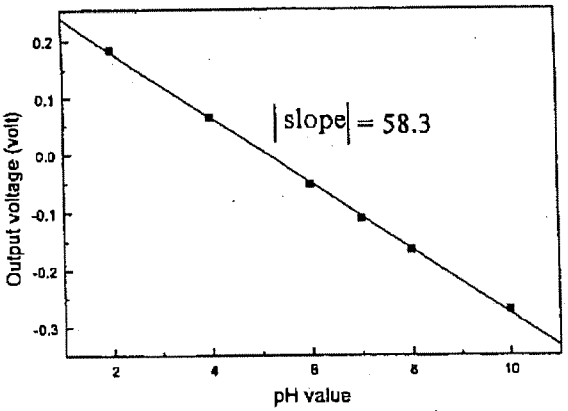

Fig. 3. Output voltage vs. pH value for the biomembrane/SnO_2/ITO glass sensing gate connected with instrumentation amplifier LT1167.

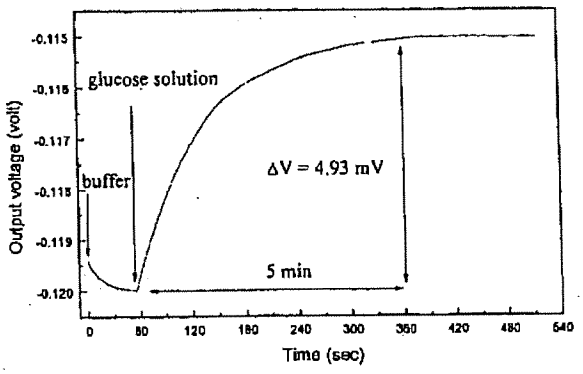

Fig. 4. Response of the separative structure of the ENFET without outer BSA membrane to detect 40 mg/dl glucose in pH 7.2 buffer solution. MnO_2 was immobilized in GOD layer.

Saito et al. employed the glucose ENFET with external BSA membrane, which is very similar our device, shows a good linear relationship with up to 300 mg/dl glucose concentration [7] which the experiments are performed in a

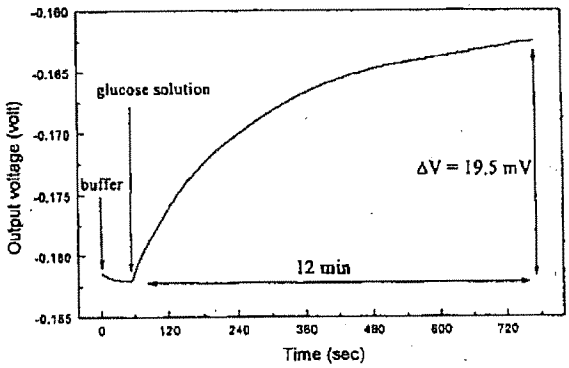

Fig. 5. Response of the separative structure of the ENFET with outer BSA membrane to detect 45 mg/dl glucose in pH 8.5 buffer solution. MnO_2 was immobilized in BSA layer.

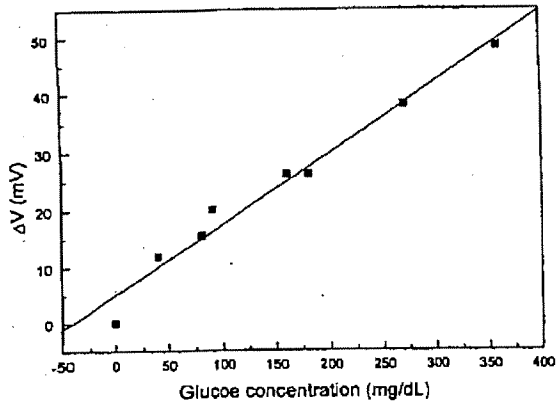

Fig. 6. Calibration curve for the glucose ENFET with outer MnO_2-doped BSA membrane. Sensors were measured in pH 8.1, 5 mM buffer solution.

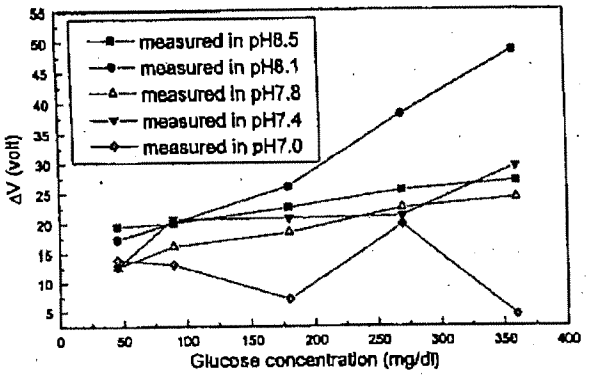

Fig. 8. Effect of pH on the glucose ENFET with outer MnO_2-doped BSA membrane. The curves correspond to different pH values of measured environments: (■) pH 8.5; (●) pH 8.1; (▲) pH 7.8; (▼) pH 7.4; (◆) pH 7.0.

stirring status. But, all the measurements in this paper are performed in a steady status. Fig. 7. shows the sensor response for sensors with non-doped MnO_2 outer BSA layer and MnO_2-doped BSA layer. The results show that sensors with MnO_2-doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer. The sensors of non-MnO_2-doped show a high response in lower glucose concentration, but very low response in high glucose concentration.

3.2. Effect of pH on the ENFET response

According to the report of Zheng and Guo, in the experiment of potentiometric determination of hydrogen peroxide at MnO_2-doped carbon paste electrode, while the pH changed in the range 7.0–8.0, the potential response increased with increasing pH [9]. The results may be related to the enhancing of oxidizing ability of H_2O_2 when pH changed in this range. For pH values in the range of 8.0–9.0, the response was almost constant. In our research, the effect of pH on the glucose ENFET response shows the best results of sensitivity and linearity that are shown in Fig. 8. As the GOD catalyzes glucose, the actual pH value of the ENFET biomembrane is lower than the pH value of buffer. The output signal of the measurement in pH 8.5 is lower than that of in pH 8.1 which is caused by that the activity of the GOD is bad in alkali [13,14]. In addition, the results measured in lower pH environments show a bad linearity which is caused by that the MnO_2 shows lower catalysis ability in acid.

3.3. Effect of MnO_2 doping position

As mention before, while the pH changed in the range 7.0–8.0, the catalysis ability of MnO_2 increased with increasing pH value. However, the actual pH value is different in individual biomembrane layer as the ENFET dips into glucose solution. The GOD layer shows the lowest pH that caused by the glucose catalyzed and producing H^+-ion. The H^+ will diffuse into the activation layer and outer layer, which close to the SnO_2 sensitive film and pH-buffer

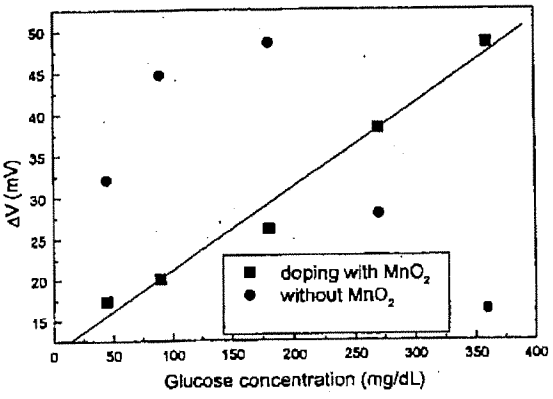

Fig. 7. Calibration curve for the glucose ENFET sensors with (●) non-doped MnO_2 outer BSA layer and (■) MnO_2-doped BSA layer. Sensors were measured in pH 8.1, 5 mM buffer solution.

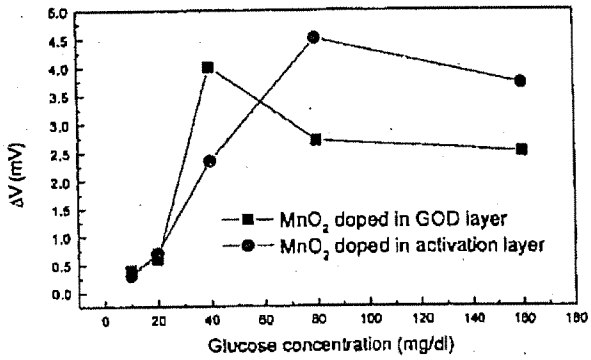

Fig. 9. Glucose concentration response of the ENFET that MnO_2-doped in the GOD layer and in the activation layer, respectively.

solution, respectively. By the effect of the carrier-mediated transport of protons, the outer membrane will show the highest pH value, which is better suitable condition for the reaction between MnO_2 and H_2O_2. As the results shown above, the glucose ENFET that biomembrane with outer MnO_2-doped BSA membrane has a linear dynamic range to 360 mg/dl. Fig. 9. shows the glucose response of MnO_2-doped in the activation layer and the GOD layer. The responses are limited in the high glucose concentration, which caused by that the reaction between MnO_2 and H_2O_2 was blocked in acid environments, especially the response of the device was MnO_2-doped in enzyme layer.

4. Conclusions

A glucose ENFET based on a new principle, in which the biomembrane was doped with MnO_2 powder. MnO_2 was used to perform the catalysis of hydrogen peroxide (one of the by-products of glucose oxidation), was proposed and its characteristics were investigated. The sensor shows a wide dynamic range to the glucose concentration of 360 mg/dl. Both the pH value of buffer solution and MnO_2-doped position affect the response of the glucose ENFET. For the MnO_2-doped effect, both the responses of MnO_2-doped in the activation layer and the GOD layer are limited in the high glucose concentration, which is caused by that the reaction between MnO_2 and H_2O_2 blocked in acid environments, especially the response of the device that MnO_2-doped in the enzyme layer. For the effect of pH value of buffer solution, the glucose ENFET, which MnO_2-doped in the outer BSA layer, measured in pH 8.1 has the largest response and the widest dynamic range in our experiments. In addition, the sensors with MnO_2-doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer.

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References

[1] S. Caras, J. Janata, Field effect transistors sensitive to penicillin, Anal. Chem. 52 (1980) 1935–1937.
[2] H.I. Seo, C.S. Kim, B.K. Sohn, T. Yeow, M.T. Son, M. Haskard, ISFET glucose sensor based on a new principle using the electrolysis of hydrogen peroxide, Sens. Actuators B 40 (1997) 1–5.
[3] A.A. Shul'ga, A.C. Sandrovsky, V.I. Strikha, A.P. Soldatkin, N.F. Starodub, A.V. El'skaya, Overall characterization of ISFET-based glucose biosensor, Sens. Actuators B 10 (1992) 41–46.
[4] B.H. van der Schoot, P. Bergveld, ISFET based enzyme sensors, Biosensors 3 (1987/88) 161–186.
[5] M. Sudoh, A. Mori, H. Fujimura, H. Katayama, Development of novel micro-sensor of disposable chips with oxygen enrichment by pre-electrolysis for monitoring blood glucose, Electrochimica Acta 44 (1999) 3848–3869.
[6] C.H. Lee, H.I. Seo, Y.C. Lee, B.W. Cho, H. Jeong, B.K. Sohn, All solid type ISFET glucose sensor with fast response and high sensitivity characteristics, Sens. Actuators B 64 (2000) 37–41.
[7] A. Saito, S. Miyamoto, J. Kimura, T. Kuriyama, ISFET glucose sensor for undiluted serum sample measurement, Sens. Actuators B 5 (1991) 237–239.
[8] A.A. Shul'ga, M. Koudelka-Hep, N.F. de Rooij, Glucose-sensitive enzyme field effect transistor using potassium ferricyanide as an oxidizing substrate, Anal. Chem. 66 (1994) 205–210.
[9] X. Zheng, Z. Guo, Potentiometric determination of hydrogen peroxide at MnO_2-doped carbon paste electrode, Talanta 50 (2000) 1157–1162.
[10] Li Te Yin, Jung Chuan Chou, Wen Yaw Chung, Tai Ping Sun, Shen Kan Hsiung, Separative structure ISFETs on a glass substrate, in: Proceeding of the International Symposium on Photonics and Applications, Nanyang Technological University, Singapore 3987 (1999) 416–419.
[11] M. Kawakami, H. Koya, S. Gondo, Immobilization of enzyme to platinum electrode and its use as enzyme electrode, Appl. Biochem. Biotechnol. 28/29 (1991) 211–219.
[12] S.K. Beh, G.J. Moody, J.D.R. Thomas, Effect of pre-treatment of platinum for modified platinum wire glucose oxidase amperometric electrodes, Analyst 114 (1989) 29–32.
[13] V. Bulmus, H. Ayhan, E. Piskin, Modified PMMA monosize microbeads for glucose oxidase immobilization, Chem. Eng. J. 65 (1997) 71–76.
[14] T. Godjevargova, V. Konsulov, A. Dimov, Preparation of an ultrafiltration membrane from the copolymer of acrylonitrileglycidyl-methacrylate utilized for immobilization of glucose oxidase, J. Membr. Sci. 152 (1999) 235–240.

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